Mediators, mechanisms and biomarkers of skeletal muscle wasting and function in cancer cachexia

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Declaration

The thesis composition herein is my own. Where I have been a member of a research group, I have made a substantial contribution to the work, and this contribution has been asserted clearly in the text. Any contribution made by others to the work is acknowledged in the text. This thesis has not been submitted for any other degree, postgraduate diploma or professional qualification.

Richard JE Skipworth

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Acknowledgements

"All things good to know are difficult to learn." □*Greek Proverb*

It is difficult to summarise the last five years in a few words, but importantly, they have served as an educational foundation in translational and clinical research, and they have confirmed, in my mind, the chosen path for my future career. There have been several successes, and there have also been a few spectacular failures. But, hopefully, when the sums are added up, the successes outnumber the failures.

There are a number of people that I must thank: first and foremost, my two long-suffering and supportive supervisors, Professor Ken Fearon and Professor Jim Ross. They have taught me everything I know about scientific methodology, scientific writing, and politics. Their constant high standards are something that I aim to maintain throughout the rest of my professional life. I would also like to thank Professor James Garden for his encouragement and for the BUPA RMO post which allowed me this opportunity in the first place.

Secondly, I have been fortunate enough to network with a number of distinguished international collaborators, and my gratitude is extended to both them and their respective research teams: Vickie Baracos (Canada); Sam Breit (Australia); Denis Guttridge (USA); Anne Voss (USA); Mike Tisdale (Aston); Simon Paterson-Brown, Grant Stewart, Catharine Sturgeon, Marie Fallon, Andy Cronshaw and Richard Anderson (all Edinburgh). I must also acknowledge the funding bodies that made this work possible: the University of Edinburgh and the Royal College of Surgeons of Edinburgh.

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Lastly, I would like to dedicate this thesis to all of the patients who took part in the studies herein. During an extremely difficult time in their lives, they embraced these projects, and even welcomed me as a friend into their homes. Unfortunately, many of these people have now passed away. My heartfelt gratitude goes out to them.

Abstract

Cachexia is a syndrome of progressive nutritional depletion that occurs in up to one-half of all patients with cancer. In particular, cachexia involves a severe and specific loss of skeletal muscle, which is associated with worsened patient morbidity, reduced quality of life (QoL), and increased mortality. The aims of this thesis were to investigate specific mediators, mechanisms and biomarkers of muscle wasting and function in cancer cachexia. Models included human cancer cell lines and tissue samples, and patients with different types of upper gastrointestinal (GI) cancer. Upper GI cancer was chosen as the model for investigation as such patients are known to have a high prevalence of cachexia.

Tumour-derived cachectic mediators were investigated initially (Chapters 3 to 5). There has been much speculation about whether the murine tumour-derived cachectic mediator known as proteolysis-inducing factor (PIF) has a human homologue, and how this may be derived from a larger gene known to encode for dermcidin, an anti-microbial peptide found in sweat. Messenger ribonucleic acid (mRNA) expression levels of dermcidin were analysed in human tumour tissue and human cancer cell lines. Expression of dermcidin was highly variable in human tumours, but appeared to be largely absent or expressed at only very low levels in oesophago-gastric cancer and prostate cancer (Chapter 3). Additionally, although human cell lines did express dermcidin mRNA, sequence analysis of complementary DNA (cDNA) derived from this mRNA demonstrated that transcripts lacked a site for the glycosylation critical to the proteolysis-inducing

activity of murine PIF, thus raising doubt about the existence of human PIF (Chapter 4).

Attention was then focused on plasma concentrations of macrophage inhibitory cytokine-1 (MIC-1), another potential tumour-derived mediator of cachexia in humans (Chapter 5). In a cohort of patients with oesophago-gastric cancer (n=293), although MIC-1 concentrations correlated with systemic inflammation (a key component of the cachexia syndrome), there was no independent link between plasma MIC-1 and nutritional status or survival. Thus, further investigations are required before MIC-1 can be determined as a key mediator of cancer cachexia in humans.

Next, the role of circulating neuroendocrine mediators, specifically sex steroids and gonadotropins, were investigated in a cohort of patients with advanced pancreatic cancer (n=175) (Chapter 6). Testosterone is known to be a significant determinant of muscle mass. However, 73% of male cancer patients were hypogonadal (as defined by calculated serum free testosterone). Hypogonadal males demonstrated shortened survival compared with eugonadal males. Furthermore, male opioid use (an iatrogenic cause of male hypogonadism) was associated with shortened survival. In contrast, 18% of postmenopausal females exhibited premenopausal or 'hyperoestrogenic' serum oestradiol levels. Hyperoestrogenic females demonstrated shortened survival compared with eugonadal females. Thus, clinical trials of androgen replacement therapy (particularly in the presence of opioid analgesia)

may be a potential route forwards in males with non-hormone sensitive types of cancer.

Regulatory mechanisms of protein degradation within skeletal muscle were then examined (Chapters 7 and 8). It has been suggested that there may be parallels in the mechanisms of muscle wasting observed in genetic disorders, such as the muscular dystrophies, and the mechanisms observed in acquired conditions such as cancer cachexia. In rectus abdominis muscle samples from patients with oesophago-gastric cancer (n=27), deregulation of the sarcolemma-bound, muscular dystrophy-associated dystrophin glycoprotein complex (DGC) (involving reduced expression of dystrophin and hyperglycosylation of key DGC proteins) was found in 17/27 (63%) samples (Chapter 7). Moreover, the presence of DGC deregulation was associated with worsened patient function and shortened patient survival. These results suggest a possible common pathway between genetic and acquired forms of muscle wasting.

The mass of a muscle is determined by the balance between protein synthesis and degradation. Pathways that can both increase protein degradation and suppress synthesis could have a profound influence on the rate of muscle wasting. Levels of the activated (phosphorylated) forms of the dsRNA-dependent protein kinase (PKR) and eukaryotic translation initiation factor 2α (eIF2α), intracellular mediators in pathways leading to increased muscle protein degradation and reduced protein synthesis, were determined in rectus abdominis samples from oesophago-gastric cancer patients (n=15) (Chapter 8). Levels of both phospho PKR and phospho

eIF2α were significantly enhanced in muscle from cachectic patients, and there was a linear relationship between myosin expression and the extent of eIF2α phosphorylation. These results suggest that phosphorylation of PKR may be an important initiator of muscle wasting in cancer cachexia.

Next, the presence of urinary biomarkers of skeletal muscle wasting were analysed using mass spectrometry in urine samples from cachectic oesophago-gastric cancer patients (n=8), weight-stable cancer patients (n=8) and healthy controls (n=8) (Chapter 9). The identification of early biomarkers of cachexia, the clinical detection of which would allow the institution of prophylactic therapeutic measures, is one of the key areas of need in cancer cachexia research. The number of protein species identified in urine samples from cachectic cancer patients (n=199) was significantly greater than that identified in samples from weight-stable cancer patients (n=79) and controls (n=49). Proteins identified specifically in cachectic samples, and thus potential biomarkers of muscle wasting that could be utilised in future clinical trials, included muscle (myosin species), cytoskeletal, and microtubule-associated proteins.

Lastly, the use of objective assessment of physical activity as a potential biomarker of skeletal muscle function, and thus a novel outcome measure in the clinical management of advanced cancer patients, was explored (Chapters 10 and 11). Criterion-based validation of estimates of energy expenditure using an accelerometer-based activity-monitoring system (activPAL™) was performed using a combination of doubly labelled water and indirect calorimetry in cancer patients

(n=6) and healthy controls (n=9) (Chapter 10). Although absolute errors for activPAL™-derived estimates of mean energy expenditure of activity (1.4%) and mean total energy expenditure (0.4%) were low, there was considerable variability in individual patient results, suggesting that further prospective validation studies of energy expenditure are required. (Step count and other PA measures have been validated previously). The activPAL™ meter was also used to take sequential measurements of objective PA in a cohort of patients with upper GI cancer undergoing palliative chemotherapy (n=16) (Chapter 11). Recruited patients demonstrated a complex "journey" of PA during treatment, with individuals describing worsened, improved or unchanged PA. However, the overall trend was one of deterioration in PA during chemotherapy. Importantly, objective measures of PA correlated with subjective scores of QoL, fatigue and performance status, supporting the use of objective PA as a patient-centred outcome.

In summary, certain circulating mediators (sex steroids) and intracellular mechanisms (DGC deregulation and PKR phosphorylation) have been shown to be important in the aetiology of cachexia/skeletal muscle wasting in cancer, and the determination of patient outcome. Urinary biomarkers of muscle protein degradation (myosin species) and biomarkers of muscle function (physical activity) may be useful as inclusion criteria and/or outcome measures in clinical trials of anti-cachexia therapies.

Abbreviations

aa, amino acids
AA, arachidonic acid
ACE, angiotensin-converting enzyme
ACN, acetonitrile
ACS, anorexia-cachexia syndrome
ACTH, adrenocorticotrophic hormone
AGP, α1-acid glycoprotein
AgRP, agouti-related peptide
AIDS, acquired immune deficiency syndrome
AJCC, American Joint Committee on Cancer
AML, acute myeloid leukaemia
AMV, avian myeloblastosis virus
ANOVA, univariate Analysis of Variance
ANS, autonomic nervous system
AP-1, activator protein-1
APC, adenomatosis polyposis coli
APPR, acute phase protein response
AR, androgen receptor
ARC, arcuate nucleus
ARE, adenylate- and uridylate-rich element
ATP, adenosine-5'-triphosphate
AU-rich, adenylate- and uridylate-rich
BAPTA/AM, 1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetra (acetoxymethyl) ester
BAT, brown adipose tissue
BCAA, branched-chain amino acid
BIA, bio-impedance analysis
BMD, Becker muscular dystrophy
BMI, body mass index
BMR, basal metabolic rate
bp, base pairs
BPAG1, bullous pemphigoid antigen 1
BPH, benign prostatic hypertrophy
BSA, bovine serum albumin
C-26, colon-26 adenocarcinoma mouse model
CA19-9, carbohydrate antigen 19-9
CAMK, Ca^{2+}/calmodulin-dependent protein kinase
cDNA, complementary deoxyribonucleic acid
CF-IRMS, continuous flow isotope ratio mass spectrometry
cFT, calculated free testosterone
CHAPS, 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulphonate
CHCA, α-cyano-4-hydroxycinnamic acid
CHF, chronic heart failure
CHO, Chinese hamster ovary
CI, confidence interval
CK, creatine kinase

CMD, congenital muscular dystrophy
COPD, chronic obstructive pulmonary disease
COX, cyclooxygenase
CRF, chronic renal failure
CRF, corticotropin releasing factor
CRP, C-reactive protein
CRUK, Cancer Research UK
CT, computerised tomography
C_T, cycle threshold
cTNM, clinical TNM staging system
CV, coefficient of variation
CYS, cysteine
DCD, dermcidin
DEXA, dual-energy x-ray absorptiometry
DG, dystroglycan
DGC, dystrophin glycoprotein complex
DHPR, dihydropyridine receptor
DHT, dihydrotestosterone
DLW, doubly labelled water
DM, diabetes mellitus
DMD, Duchenne muscular dystrophy
DNA, deoxyribonucleic acid
DNAse, deoxyribonuclease
dNTP, deoxyribonucleotide triphosphate
dp, decimal places
DSEP, diffusible survival evasion peptide
dsRNA, double-stranded ribonucleic acid
DTT, dithiothreitol
EBD, Evans Blue Dye
ECACC, European Collection of Cell Cultures
ECL, enhanced chemiluminescence
ECM, extracellular matrix
ECOG, Eastern Co-operative Oncology Group
ECRS, Edinburgh Clinical Risk Score
ECW, extracellular water
EDTA, ethylenediaminetetraacetic acid
EE, energy expenditure
EEA, energy expenditure of activity
eeF2K, eukaryotic elongation factor-2 kinase
eIF, eukaryotic initiation factor
eIF-BP, eukaryotic initiation factor binding protein
ELISA, enzyme-linked immunosorbent assay
EORTC, European Organisation for Research and Treatment of Cancer
EPA, eicosapentaenoic acid
EPO, erythropoietin
EPCRC, European Palliative Care Research Collaborative
ERK, extracellular signal-related kinase
ESPEN, European Society for Clinical Nutrition and Metabolism

ev, empty vector
FAACT, Functional Assessment of Anorexia/Cachexia Therapy
FACIT, Functional Assessment of Chronic Illness Therapy
FACIT-F, Functional Assessment of Chronic Illness Therapy - Fatigue
FCS, foetal calf serum
FDA, Food and Drug Administration
FET, Fisher's Exact test
FFM, fat-free mass
FKRP, fukutin-related protein
FOXO, Forkhead box, class O transcription factor
FSH, follicle-stimulating hormone
FT, Friedman test
5-FU, 5-fluorouracil
FWB, Functional Well-Being
GABA, gamma aminobutyric acid
GABRAPL1, gamma aminobutyric acid receptor-associated protein-like 1
GC, glucocorticoid
GCN2, general control non-repressed 2
GCR, glucocorticoid receptor
GDP, guanosine diphosphate
GDF11, growth differentiation factor 11
GF, growth factor
GFP, green fluorescent protein
GH, growth hormone
GHRP-2, growth hormone releasing peptide 2
GHS-R, growth hormone secretagogue receptor
GI, gastrointestinal
GPS, Glasgow Prognostic Score
GnRH, gonadotropin-releasing hormone
GOJ, gastro-oesophageal junction
Grb2, growth factor receptor-bound protein 2
GTP, guanosine triphosphate
HADS, Hospital Anxiety and Depression Scale
HADS-A, Hospital Anxiety and Depression Scale - Anxiety
HADS-D, Hospital Anxiety and Depression Scale - Depression
HCAP, human cachexia associated protein
HDAC5, histone deacetylase 5
15-HETE, 15-hydroxyeicosatetraenoic acid
HIF, hypoxia inducible factor
HIV, human immunodeficiency virus
HMB, β-hydroxy-β-methylbutyrate
HNSCC, head and neck squamous cell carcinoma
HPLC, high performance liquid chromatography
HR, hazards ratio
HRE, hormone response element
Hz, hertz
ICR, (Canadian) Institute for Cancer Research
ICU, intensive care unit

ICW, intracellular water
IFCC, International Federation of Clinical Chemistry and Laboratory Medicine
IFN, interferon
Ig, immunoglobulin
IGF, insulin-like growth factor
IGFBP, insulin-like growth factor binding protein
IHC, immunohistochemistry
IKK, IκB kinase
IL, interleukin
IQC, internal quality control
IQR, interquartile range
IRS, insulin receptor substrate
IV, intravenous
Kb, kilobase
kcal, kilocalorie
kDa, kiloDalton
KM, Kaplan-Meier plot
KPS, Karnofsky performance score
KWT, Kruskal-Wallis test
LARGE, like-glycosyltransferase
LBM, lean body mass
LCM, laser capture microdissection
LC-MS/MS, liquid chromatography tandem MS
LGMD, limb girdle muscular dystrophy
LH, luteinising hormone
LHRH, luteinising hormone-releasing hormone
LIF, leukaemia inhibitory factor
LLC, Lewis lung carcinoma
LMF, lipid mobilising factor
LOA, limits of agreement
LOX, lipooxygenase
LSB, Laemmli sample loading buffer
LVF, left ventricular failure
MA, megestrol acetate
mAb, monoclonal antibody
MAC, mid-arm circumference
MAC16, murine adenocarcinoma 16 mouse model
MACF1, microtubule-actin crosslinking factor
MAFbx, muscle-specific F-box, also known as atrogin-1
MALDI-TOF MS, matrix-assisted laser desorption/ionisation time-of-flight MS
MAMC, mid-arm muscle circumference
MAP1B, microtubule-associated protein 1B
MAPK, mitogen-activated protein kinase
MCA, methylcholanthrene
MC-R, melanocortin receptor
MEF2, myocyte enhancer factor-2
MET, metabolic equivalent
Met-tRNA, methionyl transfer-RNA

mGPS, modified Glasgow Prognostic Score
MIBP1, c-myc intron-binding protein 1
MIC-1, macrophage inhibitory cytokine-1
MIF, macrophage migratory inhibitory factor
MNA, Mini-Nutritional Assessment
MOWSE, Molecular Weight Search
MRC, Medical Research Council
MRI, magnetic resonance imaging
mRNA, messenger ribonucleic acid
MS, mass spectrometer/mass spectrometry
MSH, melanocyte-stimulating hormone
mTOR, mammalian target of rapamycin
MURF-1, muscle-specific RING finger-1
MW, molecular weight
MWT, Mann-Whitney U-test
MyHC, myosin heavy chain
N, Newton
N/A, not applicable
NAC, N-acetyl-L-cysteine
NCAM, neural cell adhesion molecule
NCBI, National Center for Biotechnology Information
NCCTG, North Central Cancer Treatment Group
NCI, National Cancer Institute
NCRI, National Cancer Research Institute
NF-κB, nuclear factor-κβ
NMES, neuromuscular electrical stimulation
nNOS, neuronal nitric oxide synthase
NO, nitric oxide
NPY, neuropeptide Y
NS, not significant
NSAID, non-steroidal anti-inflammatory drug
NSCLC, non-small cell lung cancer
OGC, oesophago-gastric cancer
$p70^{s6k}$, p70 s6 kinase
PA, physical activity
pAb, polyclonal antibody
PAF, platelet activating factor
PAGE, polyacrylamide gel electrophoresis
PAL, physical activity level
PAQ, physical activity questionnaire
PARP, poly (adenosine diphosphate-ribose) polymerase
PBMC, peripheral blood mononuclear cell
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PG-SGA, Patient-Generated Subjective Global Assessment
PF, physical functioning
PI3K, phosphatidylinositol 3-kinase
PIF, proteolysis-inducing factor

PIF-CP, proteolysis-inducing factor core peptide
PINI, Prognostic Inflammatory Nutritional Index
PKB, protein kinase B
PKC, protein kinase C
PKR, double-stranded RNA-dependent protein kinase
POMC, pro-opiomelanocortin
POMT, protein O-mannosyl-transferase
POMGnT, protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase
PMSF, phenylmethanesulphonylfluoride
PNGase F, N-glycosidase F
ppm, parts per million
PS, performance status
PTHrP, parathyroid hormone-related protein
PUFA, polyunsaturated fatty acid
PWB, physical well-being
QoL, quality of life
REE, resting energy expenditure
rhEPO, recombinant human erythropoietin
rhMIC-1, recombinant human macrophage inhibitory cytokine-1
RING, Really Interesting New Gene
RNA, ribonucleic acid
RNAi, RNA interference
RNAse, ribonuclease
RNS, reactive nitrogen species
ROS, reactive oxygen species
RPMI, Roswell Park Memorial Institute medium
rRNA, ribosomal RNA
RT, reverse transcription
RT-PCR, reverse transcription-polymerase chain reaction
SARM, selective androgen receptor modulator
SCLC, small cell lung cancer
SD, standard deviation
SDS, sodium dodecyl sulphate
SE, standard error
SEM, standard error of the mean
SFA, Simmonds Functional Assessment
SG, sarcoglycan
SGA, Subjective Global Assessment
SHBG, sex hormone binding globulin
SIG, Special Interest Group
SNP, single nucleotide polymorphism
SOD, superoxide dismutase
SPSS, Statistical Package for Social Services
STAT, signal transducer and activator of transcription
sTNFR75, soluble tumour necrosis factor receptor 75
TAE, Tris-acetate-EDTA
TAM, tumour-associated macrophage
TBE, Tris-borate-EDTA

TBS, Tris-buffered saline
TBW, total body water
TEE, total energy expenditure
TGF, transforming growth factor
T_h, T helper
THC, δ-9-tetrahydrocannabinol
TNF, tumour necrosis factor
TOI, Trial Outcome Index
TP, total protein
TPN, total parenteral nutrition
TPP, tripeptidyl peptidase
tRNA, transfer ribonucleic acid
TSF, triceps skinfold thickness
TT, total testosterone
TURP, transurethral resection of the prostate
UCP, uncoupling protein
UICC, Union Internationale Contre le Cancer
UPP, ubiquitin-proteasome pathway
USB, Universal Serial Bus
UTR, untranslated region
VAS, visual analogue scale
V-ATPase, vacuolar-type H^+-ATPase
VEGF, vascular endothelial growth factor
VHL, von Hippel-Lindau
WAT, white adipose tissue
WHO, World Health Organisation
WT, Wilcoxon signed-rank test
XMEA, X-linked myopathy with excessive autophagy
XOA, xanthine oxidase
ZAG, zinc-α-2-glycoprotein

Part A – Introduction and Methods

Chapter 1 – Introduction

1.1 Overview

The present thesis integrates a range of different studies of mediators, mechanisms and biomarkers of skeletal muscle wasting and function in cancer cachexia. The following chapter offers an overview of cancer cachexia. In particular, this chapter details the importance of skeletal muscle wasting within cachexia, and highlights the relevance of muscle wasting within the context of human disease. When discussing the aetiology of cancer cachexia, reference is made to potential therapeutic strategies where relevant.

Cachexia is a feature of many chronic disease states including cancer, chronic obstructive pulmonary disease (COPD), chronic heart failure (CHF), chronic renal failure (CRF), rheumatoid arthritis and acquired immune deficiency syndrome (AIDS). The etymological origins of the word *cachexia* are the Greek words *kakos* and *hexis* meaning "poor condition". Cachexia is characterised by a chronic wasting syndrome, involving loss of both lean body mass (LBM) and adipose tissue [1], which is resistant to conventional nutritional support [2]. In particular, there is a severe and specific loss of skeletal muscle [1]. Similar muscle wasting is also encountered in patients who are not considered to have cachexia *per se* but other pathological endocrine and musculoskeletal conditions, including Cushing's syndrome, diabetes mellitus (DM), hyperthyroidism, muscular dystrophy, muscle denervation, and physical inactivity (e.g. bed rest, fracture/joint immobilisation and spaceflight microgravity). In old age, muscle wasting is usually referred to as *sarcopenia*. The difference in nomenclature between cachexia and sarcopenia is a

reflection of differences that exist in the regulation of skeletal muscle protein degradation between various types of wasting. However, despite the large variety of initiating events and the subtle differences in molecular regulation, many of the underlying intracellular signalling pathways, target genes and effector mechanisms remain the same [3].

Cachexia occurs in up to one half of all patients diagnosed with cancer [4], and represents a significant physical and psychological burden for these individuals. The typical patient with advanced cachexia demonstrates obvious changes in body composition including severe weight loss and peripheral oedema [1]. Furthermore, he or she experiences numerous symptoms, including anorexia, early satiety, weakness and fatigue (Figure 1.1) [1]. These subjective feelings of weakness and fatigue may be the result of both physiological and psychological factors. Physiological phenomena include anaemia, the central effects of cytokine release, the side-effects of anti-cancer treatments, and phenomena within skeletal muscle similar to those observed in the process of ageing (e.g. reductions in muscle cell mass, glycogen content, and mitochondrial number [5, 6]). Psychological factors include depression, anxiety and insomnia [7].

The cumulative effects of the numerous signs and symptoms of cachexia on patient outcome are adverse and various: cachectic individuals report decreased quality of life (QoL) scores [8] and decreased levels of physical performance, and they experience increased risks of treatment failure [4] (be it chemotherapy, radiotherapy or surgery), increased risks of treatment side-effects [4], and increased

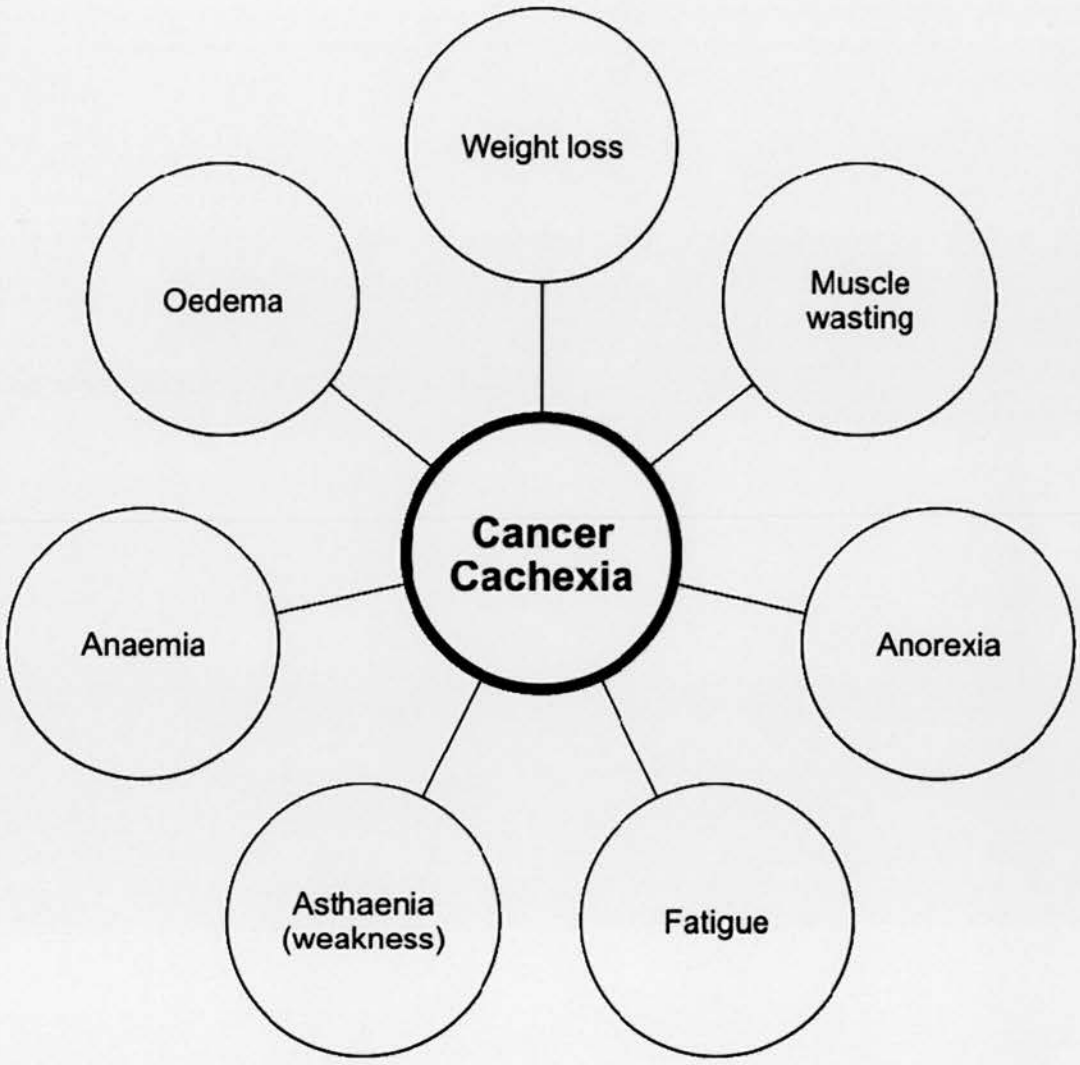

Figure 1.1 **Clinical features of cancer cachexia.**
Cancer cachexia causes a constellation of signs and symptoms within the affected patient, including changes in body composition, anaemia, and subjective feelings of fatigue.

rates of mortality [4]. In fact, it has been estimated historically that cachexia accounts for up to 20% of all cancer deaths (through immobility and cardiorespiratory failure) [9]. In cancer types that are associated with a high incidence of cachexia (e.g. advanced pancreatic cancer), the syndrome may account for up to 80% of all deaths [10, 11]. Cachexia is therefore an extremely important, yet often under-appreciated, cause of morbidity and mortality in cancer patients.

In recent years, cancer cachexia has been recognised, both nationally and internationally, as a condition that requires immediate investigation and the urgent development of a strategy for progress. Within the UK, the National Cancer Research Institute (NCRI) Strategic Planning Group on Supportive and Palliative Care has identified cachexia as a gap area that is researched inadequately at present [12]. In addition, a joint Cancer Research UK (CRUK)/Medical Research Council (MRC) workshop on cancer cachexia identified the syndrome as a key under-funded and under-researched area in cancer care [13]. Outwith the UK, the Canadian Institute for Cancer Research (ICR) has also acknowledged the need for further research. In particular, the ICR recognised that the assessment and characterisation of cachectic patient populations and the investigation of fundamental mechanisms underlying cancer cachexia are two of the most important and pressing areas requiring attention [14].

Cancer cachexia has long been considered to be the result of a variety of interactions between the host and the tumour, the full nature of which are not understood fully (Figure 1.2) [15]. The tumour's role in the aetiology of cachexia

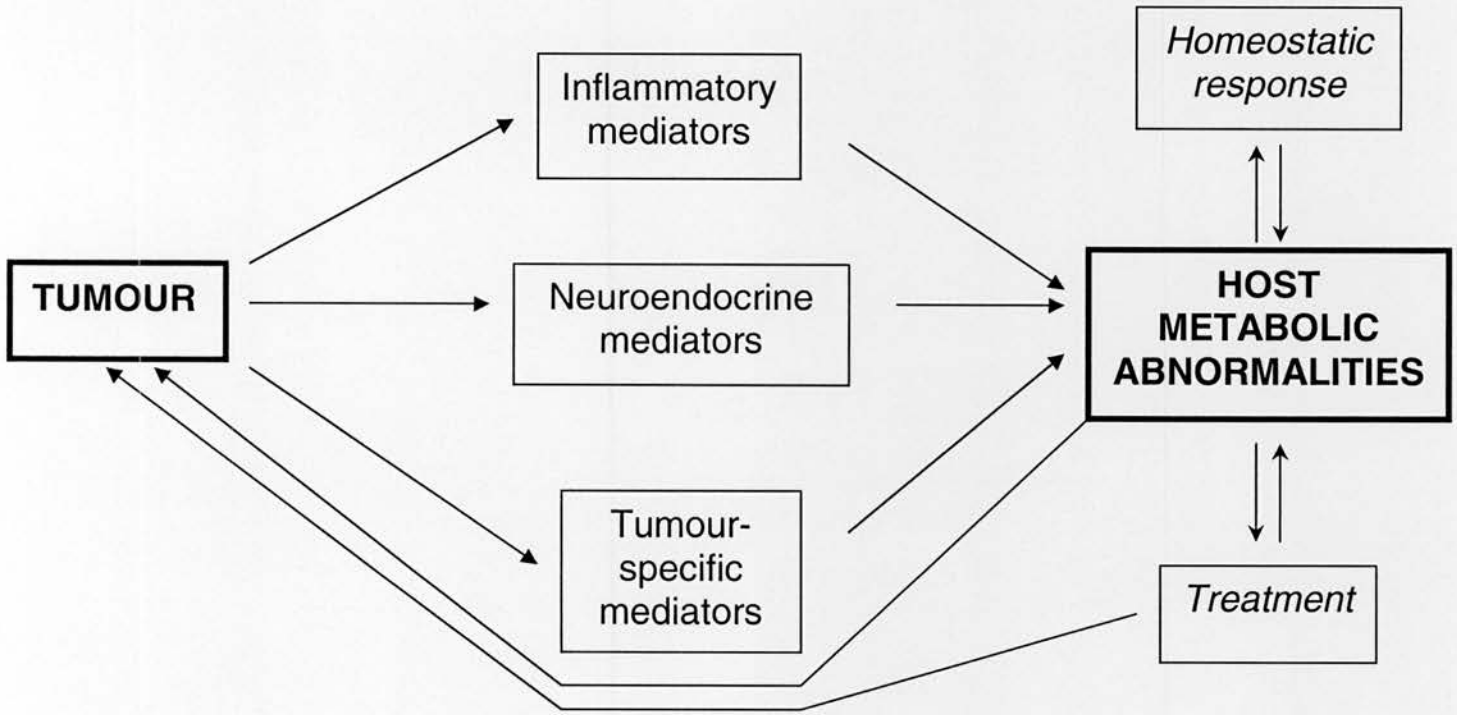

Figure 1.2 **Host-tumour interaction in cancer cachexia I.**
Different mediator pathways contribute to a variable extent within the host-tumour interaction depending, in part, on both host and tumour type. Host homeostatic mechanisms and exogenous treatments may interact further with this process.

includes the local secretion of pro-inflammatory cytokines that initiate the host systemic inflammatory response/acute phase protein response (APPR) [16], and the production of pro-cachectic factors that have direct catabolic effects on host tissues (e.g. proteolysis-inducing factor [PIF] [17] and lipid mobilising factor [LMF]) [18]. Host mechanisms involve an aberrant response to the tumour's presence, and include activation of both the APPR [16] and the neuroendocrine stress response [19, 20]. The net result of such host-tumour interaction is an alteration in body composition, a key feature of which is a severe and specific loss of skeletal muscle mass with relative preservation of the visceral protein mass [1]. These gross changes are a reflection of a complex array of metabolic changes in both the skeletal muscle and the liver of the affected individual. The key inflammatory (Chapter 1.4), neuroendocrine (Chapter 1.5) and tumour-derived mediators (Chapter 1.6) involved in cancer cachexia are summarised later in this thesis, as are the intracellular mechanisms involved in skeletal muscle protein degradation (Chapter 1.9). However, the relative importance of each of the different host/tumour mediators and mechanisms in the genesis of cachexia in individual patients/tumour types remains unclear. Moreover, in recent years, it has become apparent that host-tumour interaction may not be the sole factor driving cancer cachexia. Patient demographics, including age and habitual levels of physical activity (PA), may also play a role. Furthermore, specific patterns in the metabolism and sequestration of ingested protein by the host may account for both the irreversibility of catabolism and the sub-optimal response to nutritional support observed in cachectic cancer patients [2]. These alternative mechanisms of cancer cachexia are discussed later in this thesis (Chapter 1.8).

The previous section has afforded a general overview of cancer cachexia and has described in simple terms the mechanisms that cause cachexia. In the following section, ongoing difficulties in defining and diagnosing cancer cachexia are explained with reference to the current operational definitions and diagnostic tools available.

1.2 Definition and diagnosis of cancer cachexia

1.2.1 Definition

Cachexia is a clinical syndrome that is difficult to define as the complex, multifactorial origin of the condition precludes a uniform pathophysiological definition. Various authors have attempted to reach a concise definition for cachexia. MacDonald *et al*, for example, proposed the following definition: '*a wasting syndrome involving loss of muscle and fat directly caused by tumour factors, or indirectly caused by an aberrant host response to tumour presence*" [21]. This simple description, however, is disadvantaged, as it does not indicate the depth of complexity of cachexia. In comparison, the Cachexia Consensus Conference devised a more lengthy definition: "*cachexia is a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and hyperthyroidism and is associated with increased morbidity*"

[22]. This definition incorporates satisfactorily the complexity of cachexia but it does not estimate the expected prognosis of the cachectic patient. Any appropriate definition of cancer cachexia should attempt to identify those components of cachexia that demonstrate prognostic power and impact on important domains of QoL, physical functioning (PF) or survival. Previous attempts at tailoring the definition of cachexia according to patient outcome have yielded interesting results. In one study of weight-losing patients with unresectable pancreatic cancer (n=170), weight loss alone was not a prognostic variable and did not identify patients that differed in functional aspects of self-reported QoL or health status [8]. However, the three-factor profile of systemic inflammation (C-reactive protein [CRP] $\geq$ 10mg/l), reduced food intake ($\leq$1500kcal/day) and weight loss ($\geq$10%) defined those with both adverse function and prognosis [8].

When devising a future definition of cancer cachexia, it should also be remembered that cachexia is not a state that exists independent of time. It represents a progressive condition that can pass through early, middle and late phases (Figure 1.3) and thus any definition of cachexia or diagnostic tool should reflect temporal progression and syndrome severity. Two tools exist currently that, although not devised specifically for cachexia studies, can be used to stratify cachectic populations according to severity. The 'Constitutional Symptoms' section of the National Cancer Institute (NCI) Common Toxicity Criteria uses stratification by weight loss (Grade 0: <5%; Grade 1: 5-<10%; Grade 2: 10-<20%; Grade 3: $\geq$ 20%; Grade 4: undefined but life-threatening), whereas the Patient-Generated

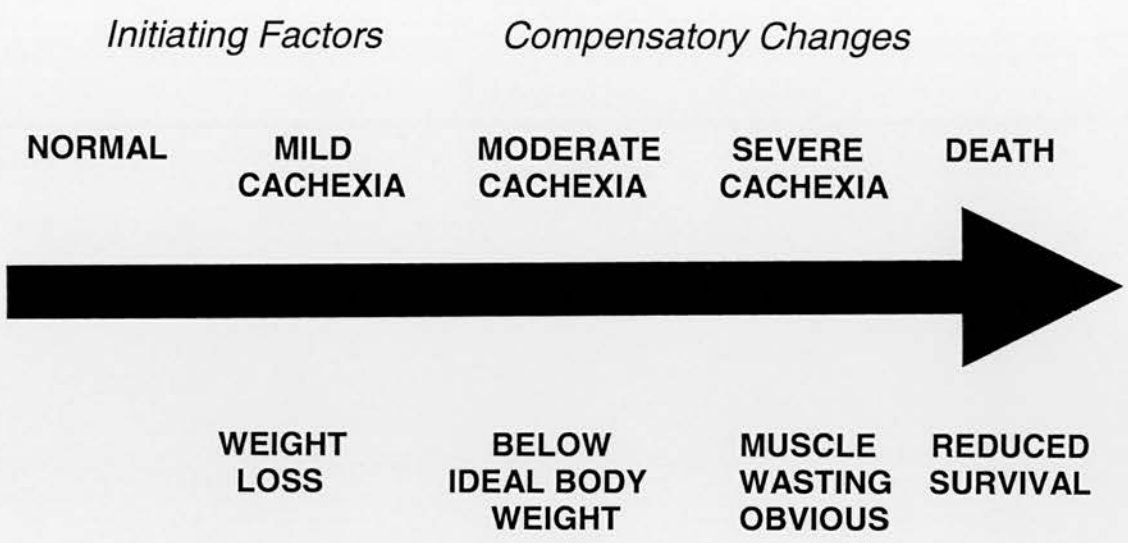

Figure 1.3 **The cancer cachexia journey.**
Cancer cachexia is a progressive condition that increases in severity over time until patient death.

Subjective Global Assessment (PG-SGA) combines questions on weight loss (graded 0–5) with food intake, functional status and other relevant factors [23]. In comparison, specific attempts to stratify cachectic cancer patients have also been carried out, and include those performed by the SCRINIO Working Group. This group employed a database of 1,307 cancer outpatients to generate a 4-stage cancer cachexia classification system ranging from asymptomatic pre-cachexia to symptomatic cachexia, based on weight loss (< or >10%) combined with the presence or absence of at least 1 symptom of anorexia, fatigue, or early satiety [24]. The concept of pre-cachexia (i.e. patients at risk of cachexia) is gaining favour in nutritional circles, as an adequate definition would allow the institution of prophylactic therapeutic intervention prior to the development of significant weight loss. Such a prophylactic approach to management is considered superior to attempting to slow or reverse nutritional depletion once weight loss is clearly evident, as such an approach is unlikely to be successful. The European Society for Clinical Nutrition and Metabolism (ESPEN) Special Interest Groups (SIGs) on "*cachexia-anorexia in chronic wasting diseases*" and "*nutrition in geriatrics*" recently defined pre-cachexia as the presence of all of the following criteria: (i) underlying chronic disease; (ii) unintentional weight loss ≤5% of usual body weight during the last 6 months; (iii) chronic or recurrent systemic inflammatory response; and (iv) anorexia or anorexia-related symptoms [25]. The European Palliative Care Research Collaborative (EPCRC) is also presently performing literature reviews and Delphi-process rounds with international cachexia experts to generate definitions for pre-cachexia, cachexia and refractory cachexia [23]. In the future, additional work should also be aimed at quantifying accurately the degree of

activation of aetiological mechanisms within cachectic patients, as identification of subgroups of cachectic patients with different dominant features might permit targeted therapeutic strategies. For example, the efficacy of anti-inflammatory agents may be influenced strongly by the level of activation of pro-inflammatory pathways (as evidenced, for example, by plasma CRP concentration).

1.2.2 Diagnosis

In the absence of a user-friendly, formalised definition, the clinical diagnosis of cancer cachexia is complicated (except when the patient has obvious severe or refractory cachexia). Severe cachexia is easily diagnosed in patients with significant non-volitional weight loss (e.g. body mass index [BMI] $<18.5 kg/m^2$ or weight loss >10% of pre-morbid weight) and muscle wasting. The choice of weight loss as a marker of cachexia is understandable as it is self-evident, simple to measure and related clinically to patient outcome [4]. However, weight loss is non-specific. Not only is weight loss compromised as a direct index of wasting in those patients who develop significant fluid retention/oedema, it may also be an insensitive marker of the physiological dysfunction driving cachexia. Furthermore, weight loss may not be helpful in identifying patients in the early stages of cachexia. Moreover, by the time a patient does manifest significant weight loss, the cachectic process is firmly established and is likely to be more difficult to slow or reverse.

The assessment of LBM may be a more sensitive measure of cachexia, as a marked loss of LBM (primarily skeletal muscle) is a specific characteristic of cachexia [26] and distinguishes the syndrome from simple starvation. However, LBM is also somewhat compromised as it includes the extracellular water (ECW) space and is therefore expanded 'artificially' by fluid retention. Recently, cross-sectional imaging of muscle mass and visceral fat (using computerised tomography [CT] or magnetic resonance imaging [MRI]) has been used as a direct clinical measure to assess cachexia [27, 28]. Although this method allows highly accurate assessments of body composition, it measures tissue volume rather than tissue content (e.g. protein) and is therefore also open to misinterpretation. However, it does have the advantage of being capable of identifying patients who, despite being over ideal weight, still have underlying evidence of skeletal muscle wasting. In one prospective CT study of a cohort of 441 patients with non-small cell lung cancer (NSCLC), mean BMI was 24.9kg/m^2, with 47.4% of patients being overweight (BMI 25-30kg/m^2) or obese (BMI >30kg/m^2), and only 7.5% being underweight (BMI <18.5kg/m^2) [29]. However, analysis of CT images demonstrated that the overall prevalence of severe muscle depletion was 46.8% and was present in patients in all BMI categories [29]. Such so-called 'overweight/obese sarcopenia' was also found in 16% of patients with pancreatic cancer, and was shown to be an independent adverse prognostic factor [30].

Compared with objective changes in body composition, subjective patient symptoms are equally compromised as diagnostic markers of cachexia. For example, fatigue may be described by 70-100% of advanced cancer patients [7],

including those who do not develop obvious wasting, and thus it retains limited sensitivity as a cachectic marker. Furthermore, patient levels of fatigue are influenced strongly by anti-cancer therapies (e.g. radiotherapy) [31]. To complicate matters further, fatigue has not yet been the subject of a consensus definition review.

The lack of strict diagnostic criteria for cachexia hinders clinical studies and has led to a multitude of inclusion criteria being used. For example, many Phase III studies (e.g. the North Central Cancer Treatment Group [NCCTG] studies) have used either involuntary weight loss (2% in 2 months or 5% in 6 months), anorexia (assessed by visual analogue scale [VAS]) or reduced oral nutritional intake (<75% of normal or <20 kcal/kg body weight) as inclusion criteria [23], whereas observational mechanistic studies have utilised different cut-offs for weight loss (e.g. >10%). Ultimately, the current *status quo* is detrimental to the progressive introduction of effective anti-cachexia therapies via clinical trials. Whichever diagnosis of cachexia is used, trial patients are only recruited once cachexia is plainly evident, and at this stage, when the patient has become severely wasted, the primary initiating events are compounded frequently by secondary factors (e.g. prolonged bed rest). In this situation, the patient can be considered to have *refractory cachexia*, as it is often impossible to make any realistic form of therapeutic intervention, either practical or (given the patient's almost imminent demise) ethically advisable. Thus, in a systematic approach to the management of cachexia, there is an urgent need for validated, internationally agreed, early biomarkers of cachexia (clinical or biochemical). Much of this thesis is targeted at

the identification of mechanisms and markers that, in the future, may be used as early biomarkers of cachexia.

In the previous section, the complexities of defining and diagnosing cancer cachexia have been discussed, and current variations in definition use within clinical trial design have been described. Moreover, the need for early markers of cachexia has been highlighted as an unmet clinical priority. In the following section, the epidemiology and incidence of cancer cachexia (within the existing limitations of definition and diagnosis) are described.

1.3 Epidemiology and incidence of cancer cachexia

Accepting the problems associated with diagnosing cachexia, the incidence of the syndrome in cancer patients is determined most readily by assessing the degree of weight loss. It is estimated that half of all cancer patients experience weight loss and one-third lose more than 5% of their original body weight [4]. In 1980, Dewys and the Eastern Co-operative Oncology Group (ECOG) performed one of the most comprehensive clinical studies to examine the prevalence and prognostic potential of weight loss in cancer patients who had not yet received chemotherapy [4]. The frequency and severity of weight loss during the previous 6 months was recorded in a range of different tumour types (Table 1.1). Importantly, the *prevalence* of weight loss in each tumour type also correlated with *magnitude* of weight loss. Upper GI

Cancer type	Percentage of patients with weight loss
Gastric cancer	85
Pancreatic cancer	83
Non-small cell lung cancer	61
Small cell lung cancer	57
Prostate cancer	56
Colon cancer	54
Unfavourable non-Hodgkin's lymphoma	48
Sarcoma	40
Acute non-lymphocytic leukaemia	39
Breast cancer	36
Favourable non-Hodgkin's lymphoma	31

Table 1.1 **Cancer types with the highest prevalence of cachexia.**
Upper GI (gastric and pancreatic) cancer and lung cancer patients, in particular, demonstrate a high prevalence of cachexia. Cachexia is defined as the presence of weight loss over the preceding 6 months (2 months for patients with pancreatic cancer). Adapted from Dewys *et al*, 1980 [4].

(gastric and pancreatic) cancer patients in particular demonstrated high frequencies of weight loss. It is worth noting that, unlike the rest of the data, weight loss in pancreatic cancer patients was only analysed over the preceding 2 months, indicating the severity of cachexia in this particular disease. In another study of 20 patients with unresectable pancreatic cancer, Wigmore *et al* demonstrated that 85% of patients had lost a median of 14% of their premorbid stable weight at diagnosis [32]. This increased to a median of 25% at or near the time of death (median inter-assessment period of 27 weeks). Over this period, LBM fell from 43.4kg to 40.1kg and adipose mass decreased from 12.5kg to 9.6kg.

Apart from patients with upper GI malignancy, lung cancer patients are the other major group known to sustain significant weight loss. In a study by Ross and co-workers, weight loss at presentation was reported by 59%, 58% and 76% of patients with small cell lung cancer (SCLC), unresectable NSCLC and mesothelioma respectively [33].

At the commencement of radiotherapy, Lees *et al* found that 57% of head and neck cancer patients had lost weight [34]. A mean weight loss of 6.5kg, equating to approximately 10% of body weight, was reported. Head and neck cancer patients suffer significantly from dysphagia and thus weight loss could be secondary to semi- starvation, rather than true cachexia. Therefore, these patients often respond readily to enteral nutritional support. However, Richey *et al* found that, following exclusion of those individuals with severe dysphagia, 46% of patients with head and neck squamous cell carcinoma (HNSCC) still experienced weight loss

exceeding 5% of premorbid body weight over the preceding 3-6 months [35].

In the previous section, the prevalence of weight loss (as a marker of cachexia) within different cancer types has been described. Patients with upper GI cancer, in particular, demonstrate high frequencies of weight loss, and for this reason, these patients (or tissue sample samples from these patients) are used as the investigative models of choice in the present thesis. In the following sections (Chapters 1.4 to 1.6), the circulating mediators that induce cachexia are discussed in more detail. Furthermore, where appropriate, inhibition or activation of specific mediators are discussed as potential therapeutic strategies against cancer cachexia. Firstly, attention is focused on inflammatory mediators and the host APPR.

1.4 Inflammatory mediators of cancer cachexia

Inflammation is of paramount importance in the aetiology of cancer cachexia. The tumour initiates a cytokine cascade that has multiple, direct, distant effects including the central induction of anorexia and the peripheral initiation of skeletal muscle protein degradation, whereas the host responds by activating the hepatic APPR (Figure 1.4). The role of cytokines in the central induction of anorexia is discussed in Chapter 1.7.

1.4.1 Pro-inflammatory and anti-inflammatory mediators

1.4.1.1 Pro-inflammatory cytokines

A variety of human cancer cell lines have been shown to produce both pro-inflammatory and anti-inflammatory cytokines [36, 37]. However, cytokines

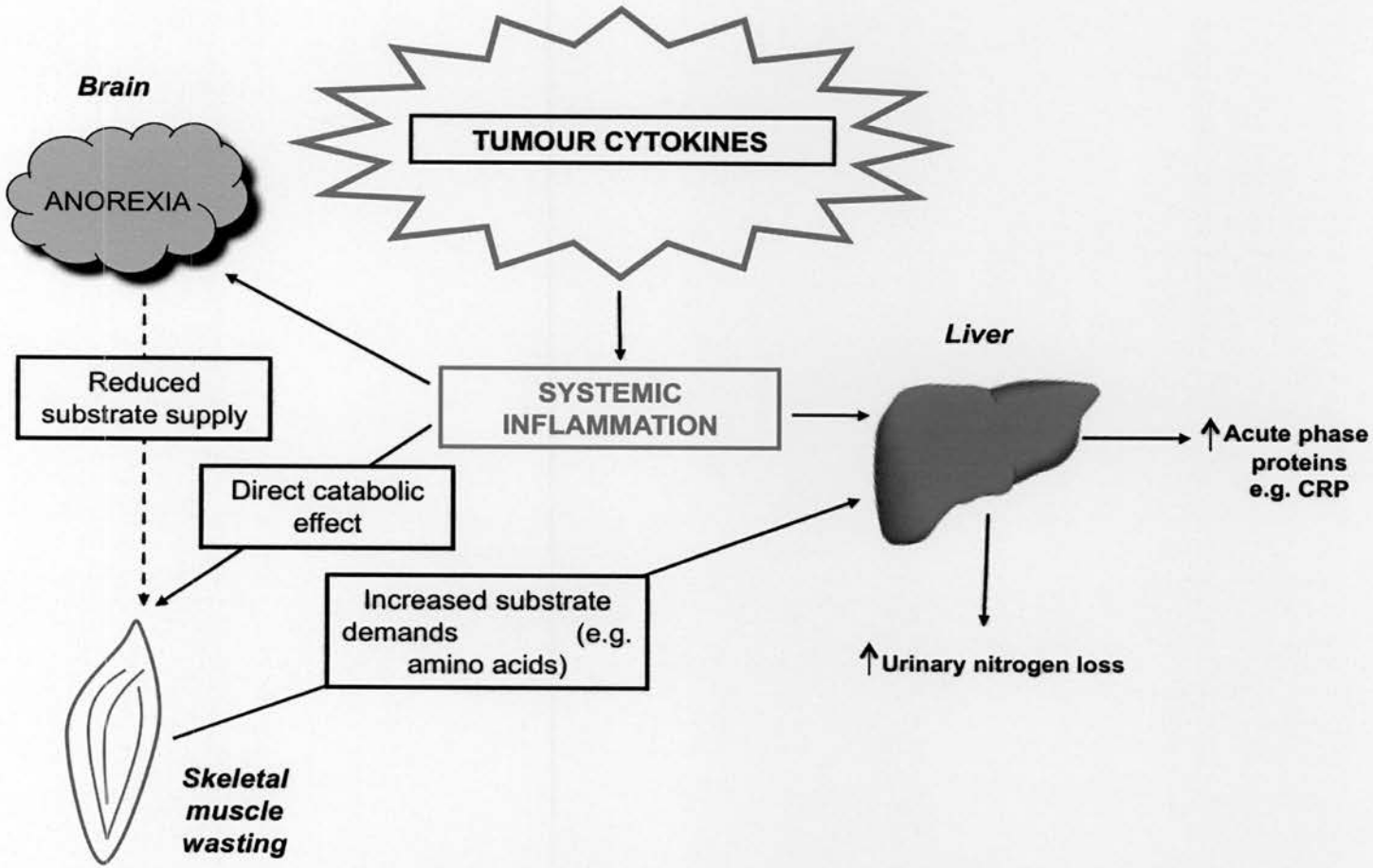

Figure 1.4 **Systemic inflammation in cancer cachexia.**
The tumour mass (i.e. host cells and tumour cells) produces cytokines that lead to a systemic inflammatory response with numerous end-organ effects, including skeletal muscle wasting, anorexia, and the induction of the hepatic APPR.

released by tumour cells *in vivo* are generally not detectable in the host's circulation, and probably act only locally to promote inflammation and activate host inflammatory cells passing through the tumour. In patients with cancer where serum levels of cytokines (e.g. tumour necrosis factor [TNF]-α) are detectable, these levels correlate poorly with patient weight loss or survival [38].

In cancer, host peripheral blood mononuclear cells (PBMCs) are recruited to tumours by various signals, including hypoxia. Thus, a major inflammatory component of tumour stroma is made up of so-called tumour–associated macrophages (TAMs). It has been postulated that the interplay between tumour cells, host lymphocytes and TAMs results in alternatively activated monocytes giving rise to a predominantly Type 2 helper T cell (T_h2) microenviroment that may favour tumour progression via the promotion of angiogenesis, the remodelling of the extracellular matrix (ECM) to allow invasion, and the suppression of adaptive immunity [39-41]. By virtue of tumour cell phenotype (production of pro-inflammatory mediators) and local tissue effects/destruction, this T_h2 type microenvironment may paradoxically promote systemic inflammation. How TAMs may be involved in the activation of host PBMCs is not yet known but macrophage-associated cytokines (e.g. macrophage migratory inhibitory factor [MIF]) have been shown to induce an APPR in isolated human hepatocytes [42]. Furthermore, high tumour chronic inflammatory cell content is associated with systemic inflammation and poor patient prognosis [16]. However, little is known about the predominant cytokine profile of tumour cells and macrophages within a cancer mass or the surrounding "normal" tissue in relation to the induction of cachexia. In oesophago-

gastric tumours, expression levels of several pro-inflammatory cytokines have been found to be elevated (e.g. interleukin [IL]-1ß) [16].

Other putative cytokine mediators of cachexia include IL-6, TNF-α, interferon (IFN)-γ, leukaemia inhibitory factor (LIF), transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF)-A and midkine. Chinese hamster ovary (CHO) cells transfected with the human TNF-α gene produced cachexia when implanted into nude mice [43]. A similar result was also found if the same mice were transfected with CHO cells constitutively producing IFN-γ [44]. An anti-IFN-γ monoclonal antibody was able to reverse the wasting syndrome associated with the murine Lewis lung carcinoma [45]. TNF-α and IFN-γ worked co-operatively to down-regulate transcription of the myosin heavy chain (MyHC) gene *in vitro* an *in vivo*, but not other core myofibrillar proteins [46]. Furthermore, MyHC protein expression was decreased in a specific fashion in the colon-26 (C-26) adenocarcinoma mouse [46], a largely IL-6 dependent model [47]. In mice with a mutation in the adenomatosis polyposis coli (APC) tumour suppressor gene, elevated circulating IL-6 levels were associated with the presence of cachexia [48]. Knockout of IL-6 prevented loss of muscle weight and epididymal fat, and reduced intestinal polyp number, implying the existence of an IL-6 cytokine amplification loop between host and tumour cells. Furthermore, recent human studies have suggested that IL-6 overexpression in weight-losing pancreatic cancer patients was related to the ability of certain IL-6-producing tumours to sensitise and induce cytokine expression in PBMCs [49].

Despite the primacy of pro-inflammatory cytokines in cancer cachexia, mediators such as IL-6, one of the key inducers of the APPR, are incapable of inducing the full cachexia syndrome alone in humans. Patients receiving treatment with IL-6 as part of antineoplastic trials report side effects of fatigue and flu-like symptoms, but only a proportion develop weight loss [50]. Therefore, it seems likely that a combination of pro-inflammatory mediators work together to induce cachexia.

1.4.1.2 Anti-inflammatory cytokines

Some cytokines may be potential repressors of cachexia. For example, IL-4, IL-10 and IL-13 all demonstrate anti-inflammatory, and hence presumably anti-cachectic, activity [51]. In the C-26 mouse model, IL-10 gene transfer reduced cachexia and prolonged survival [52]. Other cytokines (e.g. IL-15) may have potential 'antioxidant' properties that can counter the excess levels of reactive oxygen and nitrogen species (ROS and RNS) (caused by the inefficiency of host antioxidant enzymes) that have been proposed as mediators of muscle atrophy [53]. IL-15 was capable of inhibiting skeletal muscle wasting in the Yoshida AH-130 rat ascites hepatoma model by decreasing muscle protein degradation rates [54]. Overexpression of IL-15 in cultured myotubes induced a hypertrophic morphology and increased myofibrillar protein accumulation in co-cultured cells [55]. The final wasting status of the cachectic patient presumably depends on the balance between pro-inflammatory and anti-inflammatory cytokines.

1.4.1.3 Host cytokine genotype

In recent years, host cytokine genotype has been proposed as one determinant of the

development of cachexia by influencing the aetiology of systemic inflammation and the APPR [56]. In patients with oesophago-gastric cancer (OGC), specific *IL-10* and *IL-6* single nucleotide polymorphisms (SNPs) were related to systemic inflammation and adverse prognosis, and a *TNF-α* polymorphism was associated with adverse prognosis [57]. Moreover, the *TNF-α* polymorphism rs800629 was associated with anorexia in patients with NSCLC [58]. Conversely, the *IFN-γ* 'allele 2' polymorphism was related to improved survival in patients with non-resectable pancreatic cancer [59]. Regarding cachexia specifically (as evidenced by weight loss), certain *IL-1β* polymorphisms were associated with the development of cachexia and adverse prognosis in pancreatic and gastric cancer [60, 61], whereas the *IL-10* 1082 GG genotype was associated with cachexia in patients with OGC [62]. Thus, inflammation may be an inevitable consequence of cancer but the effect of this inflammation on the host may be directly related to the ability of the host to regulate such inflammation. Presumably, other aspects of skeletal muscle wasting may be influenced similarly by host genotype of other non-inflammatory cachectic mediators.

1.4.1.4 Non-cytokine inflammatory mediators

Although cytokines may play a prominent role in the activation of the APPR, other non-cytokine pro-inflammatory mediators may also exist. Elevated serum parathyroid hormone-related peptide (PTHrP) levels have been found in patients with OGC in the absence of hypercalcaemia, and were associated with the presence of an APPR and worsened survival [63]. Furthermore, studies in mice have demonstrated that the peripheral administration of PTHrP decreases food intake and

gastric emptying via the activation of hypothalamic urocortins 2 and 3 through vagal afferent pathways and the suppression of gastroduodenal motor activity [64].

1.4.1.5 Cytokine inhibitors in the treatment of cancer cachexia

Despite the pivotal role of pro-inflammatory cytokines in the aetiology of cancer cachexia, human trials to date using cytokine antagonists have not been successful at ameliorating weight loss. The use of infliximab, an anti-TNF-α monoclonal antibody (mAb), in conjunction with docetaxel was shown to be associated with increased fatigue and worsened QoL scores in NSCLC patients compared with docetaxel with placebo, and therefore the trial was stopped [65]. Neither arm of the study demonstrated palliation of weight loss. A further trial of infliximab with gemcitabine in pancreatic cancer patients was unable to demonstrate a significant improvement in LBM or survival [66], whereas a trial of etanercept, a TNF-α inhibitor, in cachectic cancer patients was not associated with weight gain or improved survival [67]. Patients receiving etanercept did, however, experience higher rates of neurotoxicity, but lower rates of anaemia and thrombocytopenia compared with placebo [67]. Trials of pentoxyfilline, a cytokine inhibitor, have also shown little clinical benefit in the treatment of cachexia and anorexia in cancer patients [68]. Human trials of an anti-IL6 mAb, in conjunction with gemcitabine, in pancreatic cancer patients are ongoing. It is anticipated that results may be superior to previous trials of cytokine inhibitors due to the critical role that IL6 plays in the initiation of the hepatic APPR (see below, p.51). Other cytokine inhibitors that have been studied in pre-clinical models of cancer cachexia, such as suramin [47], have not yet been reported in the clinical literature.

1.4.2 Host Response Mechanisms

1.4.2.1 The Hepatic Acute Phase Protein Response

An organism responds to the presence of tumour, infection, immunological disorders, tissue injury, trauma or surgery by eliciting the APPR, a response designed to help limit tissue injury. However, in certain circumstances when the APPR is prolonged or severe, it can lead to detrimental effects. The APPR is identified frequently in weight-losing cancer patients and is now well recognised as an independent adverse prognostic factor [69].

The APPR is a complex physiological event involving reprioritisation of hepatic protein synthesis, resulting in increased production of the positive acute phase proteins, such as CRP, fibrinogen, serum amyloid A, α1-acid glycoprotein (AGP), α1-antichymotrypsin and haptoglobin. The APPR is probably initiated and modulated by pro-inflammatory cytokines released from either the tumour and/or the host monocyte/macrophage cell system. Target organ changes are then mediated through secondary messengers (e.g. eicosanoids, arachidonic acid [AA], platelet activating factor [PAF], nitric oxide [NO]). The main cytokine influencing the APPR in humans is thought to be IL-6. The ability of human hepatocytes co-cultured with PBMCs to produce acute phase protein was inhibited almost completely by anti-IL-6 antibodies [70].

The mechanism by which the APPR is related to weight loss and survival is unknown, but it has been suggested that acute phase protein production represents a sink for amino acids that, in the face of inadequate dietary protein intake (due to

anorexia), contributes to the overall loss of skeletal muscle. This protein loss is further aggravated by a mismatch in the amino acid composition between proteins synthesised in the liver and those broken down in muscle [71]. It has been calculated that 2.6g of muscle protein must be catabolised to produce 1g of fibrinogen [72]. This mismatch demonstrates how aggressively the APPR may drive nutritional depletion. To complicate matters further, nutritional support, an obvious required treatment for malnourished cancer patients, may accelerate positive acute phase protein synthesis and thus exacerbate one of the basic mechanisms that contributes to the loss of lean tissue. In weight-losing patients with pancreatic cancer, synthesis rates of hepatic export proteins (fibrinogen) are elevated in the fasted state, but rise even higher during enteral feeding (Figure 1.5) [73]. These findings emphasise the need for inclusion of anti-inflammatory/anti-APPR strategies within any programme of high calorie/high protein nutritional support in the treatment of cancer cachexia.

CRP is the prototypical positive acute phase reactant (Figure 1.5), the main function of which is thought to relate to bacterial opsonisation [74]. The factors contributing to an elevated CRP may include tumour-induced systemic inflammation [16] and gut-derived inflammatory mediators as a result of increased bacterial translocation [75], although other initiatory mediators are proposed in this thesis. Elevated plasma CRP levels ($\geq$10mg/L) have been identified in patients with lung [76], breast [77], ovarian [78], renal [79], pancreatic [69], OGC [16], colorectal [80] and malignant melanoma [81] tumours, as well as individuals with multiple myeloma [82] and lymphoma [83]. Within these disease conditions, plasma CRP

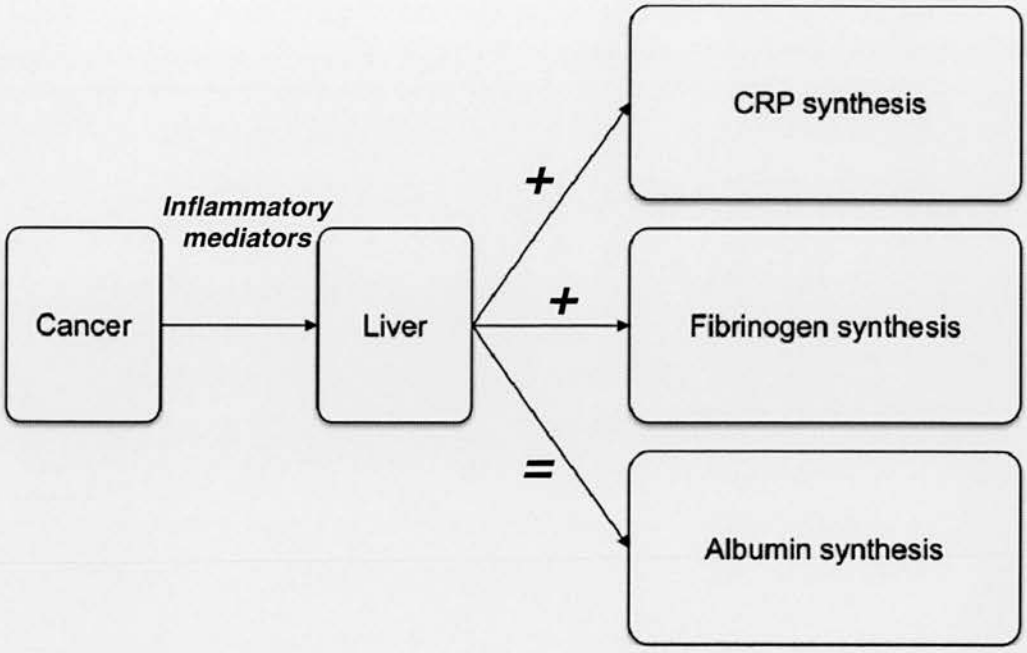

Figure 1.5 **Acute phase protein synthesis during systemic inflammation in cancer cachexia.**
Hepatic fibrinogen synthesis is increased in cachectic cancer patients and may be exacerbated further by feeding. Thus, paradoxically, feeding may contribute to the loss of lean tissue in cachexia. Although albumin levels may be decreased in cachectic cancer patients, this is not due to decreased synthesis, as synthetic levels are comparable to healthy controls.

concentration has correlated positively with weight loss [84], the presence of hypermetabolism and anorexia [85], disease recurrence [86], and reduced survival [69]. Elevated CRP levels have even demonstrated prognostic value in colorectal cancer [80], pancreatic cancer [87] and OGC patients [88] undergoing surgical resection with curative intent. For these reasons, in the present thesis, circulating CRP concentration is utilised as a measure of systemic inflammation. Regarding other positive acute phase reactants, elevated fibrinogen levels have also been associated with poor outcome in melanoma [89] and lung cancer patients [90].

In contrast to positive acute phase reactants, the plasma concentrations of other liver export proteins (the so-called negative acute phase reactants) may decrease in the cachectic patient. Albumin is one example of a negative acute phase reactant. However, despite significant hypoalbuminaemia, the total albumin synthesis rate in both the fasting and fed state is not different between patients with advanced cancer and healthy controls (Figure 1.5) [73, 91]. Thus, the hypoalbuminaemia of cancer cachexia cannot be explained by a reduced synthesis rate, but must reflect alternative mechanisms of albumin loss e.g. increased transcapillary escape secondary to an increase in microvascular permeability. When considering whole-body protein kinetics, this means that, in the cachectic patient, the increased hepatic synthesis of positive acute phase reactants is not compensated for by reduced synthesis of negative acute phase reactants, resulting in net increased hepatic protein production.

1.4.2.2 Prognostic scoring systems

Although plasma levels of acute phase reactants may exhibit prognostic value when considered singly, some authors have attempted to combine the results of different acute phase reactants or add in clinico-pathological variables to generate prognostic scoring systems for clinical decision-making. The Glasgow Prognostic Score (GPS) and modified GPS (mGPS) are simple scores based on the presence or absence of both elevated plasma CRP and hypoalbuminaemia. Both scores have demonstrated prognostic power in a range of cancer types, particularly colorectal cancer in which they have been able to predict survival even in patients undergoing surgery with curative intent [92, 93]. In comparison, the Edinburgh Clinical Risk Score (ECRS) combines CRP, weight loss, Karnofsky performance score (KPS) and clinical stage of disease (cTNM) and has been shown to predict accurately death at 12 and 24 months in patients with OGC [94]. A recent study that compared the mGPS and ECRS in OGC patients concluded that, of the 2 tests, only the mGPS was associated with survival on multivariate analysis [95]. The mGPS is utilised in the present thesis in Chapter 4.

In the previous section, the integral roles of inflammatory cytokines (particularly IL-6) and the host APPR in the aetiology of cancer cachexia have been explored. However, the present lack of efficacy of cytokine antagonists in human trials has also been described. Host cytokine genotype as a determinant of inflammation regulation has been discussed as a potential regulator of weight loss and prognosis in cancer patients. Furthermore, the mechanism by which feeding, a basic anti-cachexia therapy, can exacerbate the host APPR has been explained. In the

following section, the subject of discussion moves away from inflammatory factors to neuroendocrine mediators of cancer cachexia. Furthermore, the potential role of autonomic dysregulation within cancer cachexia is also explored.

1.5 Neuroendocrine mediators of cancer cachexia

The precise roles of circulating neuroendocrine hormones/growth factors (GFs) and the neuroendocrine stress response in the aetiology of cancer cachexia, and in particular skeletal muscle atrophy, are understood poorly. However, when considering their roles, one can regard muscle wasting as the result of an imbalance between anabolic and catabolic mediators.

1.5.1 Anabolic hormones and growth factors

1.5.1.1 Androgens

A significant portion of the anabolic action of testosterone and the other androgens occurs directly in skeletal muscle, predominantly through interactions with the androgen receptor (AR). In this mechanism, free testosterone is transported into the cytoplasm of target tissue cells, where it may bind directly to the AR, or first be reduced to 5α-dihydrotestosterone (DHT) by cytoplasmic 5α-reductase prior to receptor binding. The receptor complex then undergoes a conformational change that allows it to translocate to the nucleus and bind directly to specific deoxyribonucleic acid (DNA) nucleotide sequences, termed hormone response elements (HREs), which influence the transcriptional activity of certain pro-anabolic genes. Despite the predominance of this particular androgenic pathway, growing evidence suggests that testosterone may also exert anabolic effects through

actions mediated via the skeletal muscle *α-actin* promoter [96] and the oestrogen receptor (following the aromatisation of testosterone to oestradiol) [97], and through other indirect mechanisms. Such indirect pathways may include modulation of the growth hormone (GH)/insulin-like growth factor (IGF)-1 axis (see below, p.58) [98], regulation of motor neurons [99], and genotype-dependent changes in spontaneous PA and reactivity (animal locomotor activity in response to a novel environment) [100].

A significant prevalence of male hypogonadism has been detected in patients with metastatic cancer [101], old age [102] and human immunodeficiency virus (HIV) [103]. However, despite these observations, many authors have expressed concern regarding the lack of clear epidemiological data linking hypogonadism to the clinical and biological sequelae of cancer cachexia [104]. Furthermore, trials of androgen therapy in pre-clinical and clinical models of cancer cachexia have not been successful consistently. For example, nandrolone propionate therapy in a murine model of cancer cachexia was only able to demonstrate weight gain via fluid retention [105], whereas a trial of nandrolone decanoate in patients with advanced NSCLC could demonstrate only a trend for decreased weight loss [106]. One potential obstacle to the success of androgen therapy in cancer cachexia is that the ideal dose of androgen is currently unknown. Debate exists as to whether a physiological or supra-physiological approach is superior. However, regardless of the tempered findings in cancer cachexia, androgens, including testosterone, nandrolone and oxandrolone, are now recognised therapies for patients with HIV-associated wasting [107], sarcopenia [108], burns [109, 110], and muscle atrophy

associated with orchidectomy [111]. However, even in these patients, despite obvious gains in muscle mass, studies have not always been able to prove conclusively an improvement in patient functional status [107].

Recently, selective androgen receptor modulators (SARMs) have received much attention as potential muscle-targeted treatments for cancer cachexia. Already, such drugs have demonstrated efficacy at restoring LBM and muscle strength [112], and reducing bone loss [113] in orchidectomised male rats. In murine models, SARMs blocked dexamethasone-induced dephosphorylation of proteins involved in protein synthesis and reduced castration-induced up-regulation of E3 ligases and down-regulation of IGF-1 [114]. In humans, Phase I and II clinical trials have shown that SARMs increased LBM and enhanced functional status [115], but large, randomised controlled trials in cachectic cancer patients are still awaited.

To investigate further the epidemiology of hypogonadism in cancer cachexia and the role of androgens within the context of human muscle wasting, circulating levels of free testosterone and other sex steroids/gonadotropins in cancer patients are studied in detail later in this thesis (Chapter 6).

1.5.1.2 Growth Hormone

GH is of vital importance in promoting growth and protein synthesis in a wide range of tissues (including skeletal muscle). For example, animal studies have shown that muscles lacking GH receptors are smaller in mass due to a decrease in myofibre size and number [116]. Furthermore, GH facilitates the fusion of

myoblasts to nascent myotubes *in vitro* [117]. GH appears to achieve its actions through both IGF-1-dependent and independent actions (GH is the most potent stimulator of IGF-1 production by the liver [see below, p. 61]) [117].

Despite the importance of GH in muscle anabolism, evidence for alterations of the somatotroph axis as a causative factor in human muscle atrophy is limited. Studies in cachectic cancer patients have demonstrated high GH levels as one might expect in a normal reactive response [118]. However, in certain tumour types there may be emerging evidence of a GH-resistance syndrome [119].

Trials of GH therapy in catabolic patients have had mixed results, and, as with androgens, the exact required dosage remains unclear. GH has been used successfully to reduce muscle loss in patients with HIV-associated wasting [120] and burns [121]. However, under such circumstances, patients often experience symptoms similar to subacute acromegaly (e.g. stiff joints and carpal tunnel syndrome). Furthermore, although preliminary, small-scale studies yielded promising findings, large, randomised, placebo-controlled, multi-centre trials of GH administration to critically ill intensive care unit (ICU) patients have demonstrated an increase in morbidity and mortality [122], possibly related to GH-induced reduction in glycaemic control. Moreover, recent studies have shown that GH administration stimulates collagen synthesis in human tendon and skeletal muscle without affecting myofibrillar protein synthesis, suggesting that GH is more important in matrix strengthening than for actual muscle cell hypertrophy [123].

Thus, the efficacy of GH treatment in humans as a method of increasing skeletal muscle mass remains uncertain.

1.5.1.3 Insulin

Insulin provides an anabolic stimulus for muscle protein production. Therefore, in 'insulin deficient' states (i.e. Type I DM or insulin resistance) there is a reduction of muscle protein synthesis. Insulin resistance and impaired glucose utilisation are common findings in a wide range of diseases associated with muscle wasting, including cancer cachexia [19, 124]. For example, in CHF patients, insulin resistance is related directly to reduced muscle strength, exercise capacity and patient survival [125].

There is now robust evidence that insulin resistance also results from systemic inflammation. In weight-losing cancer patients, insulin resistance is associated with increased plasma concentrations of IL-6 [19]. Furthermore, in healthy human subjects, TNF-α has been shown to promote insulin resistance in skeletal muscle by attenuating phosphorylation of the insulin receptor substrate (IRS)-1, thus altering insulin signal transduction [126].

Low dose insulin therapy administered to weight-losing cancer patients in the context of additional palliative support (anti-inflammatory treatment with indomethacin; prevention of anaemia with recombinant erythropoietin [rhEPO]; and specialised nutritional care including oral supplements and home total parenteral nutrition [TPN]) stimulated carbohydrate intake, decreased serum-free

fatty acids, increased whole body fat, but did not affect LBM, maximum exercise capacity or spontaneous PA [127].

1.5.1.4 Insulin-Like Growth Factor-1

IGF-1 is a polypeptide hormone produced by the liver (and target tissues) under the positive regulatory control of GH. The anabolic action of IGF-1 is mediated through the tyrosine kinase IGF-1 receptor interacting with members of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. The majority of IGF-1 present in body fluids is bound to one of six structurally related high-affinity IGF binding proteins (IGFBP). These proteins can thus modulate bioavailability either positively or negatively. The catabolic response in patients is usually characterised by a marked increase in IGFBP-1, frequent decrease in IGFBP-3, and a TNF-dependent decrease in IGFBP-5 [128]. GH not only stimulates the production of IGF-1, but may also modulate IGFBP levels [129], thus further influencing IGF-1 activity.

In the rat AH-130 hepatoma ascites model of cancer cachexia, muscle expression of IGF-1 messenger ribonucleic acid (mRNA) decreased progressively whereas IGF-1 receptor and insulin receptor mRNA levels increased compared with controls [20]. Furthermore, circulating levels of IGF-1 and insulin were reduced. However, the exact mechanism of IGF-1 down-regulation in this scenario is unclear, as administration of exogenous IGF-1 to tumour-bearing rats did not prevent cachexia [20].

Despite the failure of exogenous IGF-1 in the above-mentioned study, IGF-1 upregulation may still represent a possible therapeutic mechanism for the treatment of human muscle wasting. In rodent models, transgenic overexpression of IGF-1 inhibits muscle atrophy in both chronic left ventricular failure (LVF) and angiotensin II-induced wasting [130]. Serum IGF-1 has been capable of predicting body cell mass and weight loss in cancer patients [131]. Furthermore, higher circulating levels of IGF-1 are associated with improved patient survival in cardiovascular disease [132] and cancer [133].

In the field of cancer research, much work has focused on the inhibition of the GH/IGF-1 axis as a means of decelerating tumour growth [134]. Although this work might ultimately achieve its goal, it may occur at the expense of accelerated muscle wasting.

1.5.1.5 β-adrenergic system

Although used traditionally for bronchospasm, it has become evident that some β-adrenoceptor agonists also exhibit 'repartitioning effects' within the body, resulting in an increase in muscle mass and a decrease in fat mass [135]. In animal tumour models, β-agonists have decreased muscle protein breakdown [136], increased muscle protein synthesis [137], diminished the increased rate of muscle apoptosis [138], and also induced the expression of genes involved in lipid metabolism, indicating a metabolic shift towards a more oxidative phenotype [139]. However, the long-term results of β-agonist therapy in humans remain unclear, and could be tempered by undesirable side effects [135]. Although β2-agonist administration in

the perfused hind-limb model increased gastrocnemius-plantaris-soleus muscle mass by 21% and stimulated protein expression of type IIb MyHC, greater fatigue occurred on contraction [140]. Furthermore, chronic β2-agonist therapy resulted in impaired maximal lipid oxidation capacity and reduced glutamate dehydrogenase activity, implying that the shift in muscle phenotype may not always be a beneficial one [140]. Moreover, older generation β-agonists (such as clenbuterol and fenoterol) are associated with significant cardiovascular side effects [135]. Results of Phase I/II clinical trials of formoterol (a newer generation β-agonist with a more acceptable cardiovascular side effect profile) and megestrol acetate (MA, an appetite stimulant) are awaited.

1.5.2 Catabolic hormones and growth factors

1.5.2.1 Myostatin

Myostatin, a member of the TGF-β protein superfamily, is an autocrine/paracrine inhibitory GF produced by skeletal muscle myocytes. It binds to the activin type 2 cell surface receptor to activate the SMAD (member of TGF-β superfamily of modulators) intracellular signalling pathways leading to changes in gene regulation. In particular, myostatin activity is able to repress myogenesis by inhibiting the synthesis and activity of the myogenic regulatory factor, MyoD [141].

Myostatin overexpression has been detected in a wide variety of forms of muscle wasting, including cancer cachexia [142], HIV-associated wasting, disuse atrophy and sarcopenia [143]. Furthermore, exogenous myostatin administration [144] or forced muscle-specific myostatin overexpression [145] in mice leads to dramatic

muscle wasting. Therefore, methods to inhibit myostatin expression have been proposed as potential therapies to reduce muscle wasting and promote muscle growth. Models of myostatin inhibition are known to occur both naturally and artificially. In experimental murine models, deletion of the *myostatin* gene locus resulted in a 200-250% increase in muscle mass [146]. Furthermore, loss of myostatin has been shown to improve significantly muscle mass, strength and function in the *mdx* mouse model of muscular dystrophy [147]. Pharmacological and genetic methods of myostatin inhibition have been shown to improve muscle mass in a wide range of laboratory models, including cancer cachexia [148]. Inhibitory agents have included antisense RNA oligonucleotides [149], activin receptor molecules [150] and follistatin, the natural inhibitor of myostatin [148, 151]. Recently, studies in several cancer cachexia models have shown that pharmacological blockade of ActRIIB, a high affinity activin type 2 receptor that mediates signalling by a subset of TGF-β family ligands including myostatin, activin and growth differentiation factor 11 (GDF11), reversed completely prior loss of skeletal muscle and cancer-induced cardiac atrophy [152]. Moreover, this treatment prolonged survival dramatically, even in those animals in which tumour growth was not inhibited and fat loss was not reduced [152].

Spontaneous *myostatin* gene mutations have been described in humans [153], whippets [154], and Belgian Blue and Piedmontese cattle [155]. This gene is highly conserved between species, but the evolutionary advantage of myostatin expression is not immediately obvious: loss-of-function *myostatin* gene mutations appear to confer significant strength upon the host without obvious associated survival

disadvantage. Furthermore, in whippets, a significant advantage of heterozygous *myostatin* mutation status on racing performance has also been demonstrated (although dogs with homozygous mutations were less able runners) [156].

When considering myostatin inhibition as an anti-cachexia therapy in humans, one potential complication of long-term treatment may be the depletion of muscle stem cells [157], thus significantly worsening the syndrome were disease relapse to occur. However, trials of myostatin inhibition are due to start in humans.

1.5.2.2 Glucocorticoids

In a human patient, glucocorticoids (GCs) may be endogenous (e.g. cortisol) or exogenous (i.e. medical therapy for chronic inflammatory disease) in origin. Any type of GC can cause muscle wasting, but it is much more common with fluorinated steroids, such as dexamethasone or triamcinolone, than with nonfluorinated steroids, such as prednisolone or hydrocortisone [158].

The catabolic effects of GCs on skeletal muscle appear to be mediated by several different mechanisms. Firstly, GC combines with the cytosolic GC receptor (GCR) to enter the cell nucleus and regulate gene transcription. This leads to both upregulation of protein degradation pathways and enhanced glutamine synthetase activity, resulting in increased levels of intracellular glutamine, an important substrate for gluconeogenesis and energy yielding pathways [159]. There is a subsequent reduction in the amino acid pool, thus preventing muscle protein synthesis [160]. Secondly, GCs inhibit muscle differentiation through the

upregulation of myostatin. The human *myostatin* gene contains a number of GC response elements within its promoter region, allowing dose dependent activation of myostatin production [161]. Myostatin gene deletion in mice reduced glucocorticoid-induced muscle proteolysis [162].

Hypercortisolaemia is associated with muscle atrophy in Cushing's disease, severe trauma (e.g. burns), disuse muscle atrophy [163], and cancer cachexia [164]. Weight-losing pancreatic cancer patients have been shown to exhibit elevated serum cortisol levels and cortisol: insulin ratios [19]. The muscles most commonly affected by GC-induced atrophy are the proximal limb muscles and, in particular, the quadriceps and pelvic muscles. Fast-twitch fibres are especially susceptible [158].

Several strategies for the treatment of GC-induced muscle atrophy have been proposed including recombinant human GH [165], IGF-1 [166] and anabolic steroids [112]. There have also been suggestions that progression of wasting may be slowed by exercise, as the mechanism of GC muscle wasting is similar to that of disuse atrophy [167]. Withdrawal or dose reduction of exogenous GC may give symptomatic improvement; however this may obviously result in relapse of the underlying inflammatory condition for which the steroids were prescribed.

1.5.2.3 Angiotensin II

Angiotensin II is a component hormone of the renin-angiotensin syndrome. Angiotensinogen produced by the liver is converted to angiotensin I by the action

of renin (secreted by the kidney), which is then converted to angiotensin II by the removal of 2 carboxy-terminal residues by angiotensin-converting enzyme (ACE) (found predominantly in lung capillaries). Angiotensin II may play a role in both cardiac and cancer cachexia via upregulation of the ubiquitin-proteasome pathway (UPP) (one of the key intracellular mechanisms of muscle protein degradation) [168, 169], the direct inhibition of protein synthesis [170], the modulation of IGF-1 pathways [171, 172], and the formation of ROS [173]. In animals, angiotensin II produces weight loss through a repressor-independent mechanism, accompanied by decreased levels of circulating and skeletal muscle IGF-1 and increased skeletal mRNA levels of specific E3 ubiquitin ligases [128]. The induction of the UPP may occur via a protein kinase C (PKC) and non-classical nuclear factor-κB (NF-κB)-dependent pathway [174]. Furthermore, plasma angiotensinogen levels are increased by the actions of thyroxine and corticosteroids [175], thus affording another mechanism through which these two hormones can induce muscle wasting.

Methods to reduce circulating angiotensin II levels represent possible therapeutic options for cachexia, and it has been shown that ACE inhibitors are capable of reducing the risk of weight loss in patients with CHF [176]. No trials in cachectic cancer patients have yet been published. However, in patients with newly diagnosed GI cancer and NSCLC, genetic polymorphisms in the *ACE* gene have been shown to be associated with LBM, fat mass and muscle strength [177]. Further data are required to determine the clinical impact of *ACE* polymorphisms in cachectic cancer patients.

1.5.3 Autonomic nervous system

Dysregulation of the autonomic nervous system (ANS) has been recognised in association with advanced cancer. In one study of male patients with metastatic or recurrent cancer, autonomic dysfunction was found in 81% of patients [178], whereas another study found a significant association between autonomic dysfunction and survival [179]. It has been proposed that autonomic dysfunction might actually represent a tumour-related epiphenomenon, the role of which is to promote tumour cell survival [180]. The hypothesis states that, by inducing host sympathetic activation and cytokine cascade, the tumour is capable of promoting its own growth (via adrenergic-induced angiogenesis) and evading host immunity (via cytokine and adrenergic-induced shift of local immune balance to a tumour-favourable T_h2 bias). Other researchers have also postulated that cytokine-induced anorexia/reduction in food intake may modulate both the neurohormonal and autonomic axes even further [181]. How ANS dysregulation might impact on skeletal muscle wasting, however, is not currently known.

In the previous section, the role of circulating neuroendocrine mediators in the aetiology of cachexia has been discussed, by considering skeletal muscle atrophy as a result of either decreased anabolic factors and/or increased catabolic factors. Various abnormalities in circulating neuroendocrine factors have been identified in cachectic cancer patients, but the identities of the most influential are unclear currently. Exogenous supplementation of androgens, GH and insulin, and inhibition of myostatin have been discussed as potential anti-cachectic therapeutic strategies. Furthermore, early studies exploring the significance of ANS dysregulation within

cancer cachexia have been referenced. In the following section, the subject of discussion changes once again to focus on the role of tumour-derived mediators of cachexia.

1.6 Tumour-derived mediators of cancer cachexia

The induction of cancer cachexia in surgically conjoined tumour-bearing rats provided early evidence that cachexia is mediated by circulating, tumour-derived factors that can initiate catabolism of host tissues [182]. A number of cachectic mediators, including pro-inflammatory cytokines, could be considered tumour-derived in origin. However, this section focuses predominantly on PIF and LMF.

1.6.1 Proteolysis-inducing factor

PIF was first identified as a glycosylated polypeptide using an antibody cloned from splenocytes of mice transplanted with the murine adenocarcinoma 16 (MAC16) tumour [17]. This antibody bound to a 24kDa sulphated glycoprotein, which was also present in the urine of cachectic human cancer patients [17]. A hybridoma was subsequently developed for the production of the antibody, for use in purification of PIF by affinity chromatography and for assay of physiological samples. Immunoreactivity with the murine anti-PIF antibody has since been described in the urine of weight-losing patients with carcinoma of the pancreas, breast, ovary, lung, colon, rectum and liver [17]. Patients with pancreatic cancer demonstrating PIF immunoreactivity in urine had a significantly greater total weight loss and rate of weight loss than patients whose urine did not [183].

Furthermore, isolation of protein with PIF immunoreactivity from human urine induced muscle wasting when injected into mice [184].

In murine and pre-clinical models, PIF appears to be capable of inducing skeletal muscle wasting via a number of different mechanisms. Firstly, PIF may induce muscle protein degradation via activation of the UPP. PIF-induced protein degradation, both in gastrocnemius muscle *in vivo* and murine fibroblasts *in vitro*, was associated with increased expression of mRNA and protein of the ubiquitin conjugating protein and the proteasome α and β subunits [185]. Upregulated expression of the UPP in murine myotubes was associated with PIF-induced activation of the transcription factor NF-κB [186], possibly through activation of PKC [187]. ROS may be additional intermediates of murine PIF-induced proteolysis *in vitro* [173]. Secondly, not only does PIF induce muscle protein degradation, but it also appears to inhibit muscle protein synthesis by reducing translational efficiency via double-stranded RNA-dependent protein kinase (PKR) and eukaryotic initiation factor (eIF) 2α phosphorylation [188]. Thirdly, PIF is also capable of inducing skeletal muscle apoptosis via stimulation of caspase activity *in vitro* [189, 190]. Lastly, PIF may also induce skeletal muscle wasting indirectly by virtue of pro-inflammatory activity. Protein with PIF immunoreactivity extracted from human urine has been shown to activate NF-κB and signal transducer and activator of transcription 3 (STAT3) in isolated hepatocytes, certain endothelial cell types, monocytes and Kupffer cells, resulting in increased production of IL-6, IL-8 and CRP, and decreased production of transferrin [191-193]. Thus, PIF and Il-6 may be common activators of the APPR in cancer cachexia.

PIF-like transcripts appear to be absent or minimally present in normal human tissues, with the exception of sweat glands [194], the pons, and the paracentral gyrus of the cerebral cortex [195]. In contrast, PIF-like mRNA has been demonstrated in malignant human tumours, including upper GI cancer [196], prostate cancer [197] and the human melanoma cell line, G361 [198]. Further expression studies of PIF mRNA are detailed later in this thesis (Chapters 3 and 4).

There remain, however, a number of unresolved issues concerning the biology of PIF. Firstly, it appears to be transcribed from a gene that, depending on the extent of transcription, may result in other functional proteins being produced (Figure 1.6). In 1998, Incyte Pharmaceuticals first identified the human homologue of murine PIF in a breast cancer library by using the known sequence of the 20-amino acid PIF core peptide (PIF-CP) [199]. The discovered sequence shared 90% homology with murine PIF and was named the human cachexia associated protein (*HCAP*). HCAP mRNA was later detected in prostate cancer cell lines, whereas HCAP protein was found in metastatic tissue and urine of cachectic patients with prostate cancer using rabbit antisera raised to a peptide consisting of the 25 amino acids at the N-terminus of the deduced HCAP protein that overlapped with the known sequence of PIF [197]. The gene sequence of *HCAP* is identical to that of another gene known as *DCD*. This latter gene codes for a 110-amino acid, antimicrobial protein called dermcidin, which was expressed specifically and constitutively in sweat glands [194]. Only the terminal 47 amino acids of the DCD sequence were secreted into human sweat, and this peptide fragment was named DCD-1 (Figure 1.6). Also in 1998, a 30-amino acid, survival-promoting peptide (known as Y-P30)

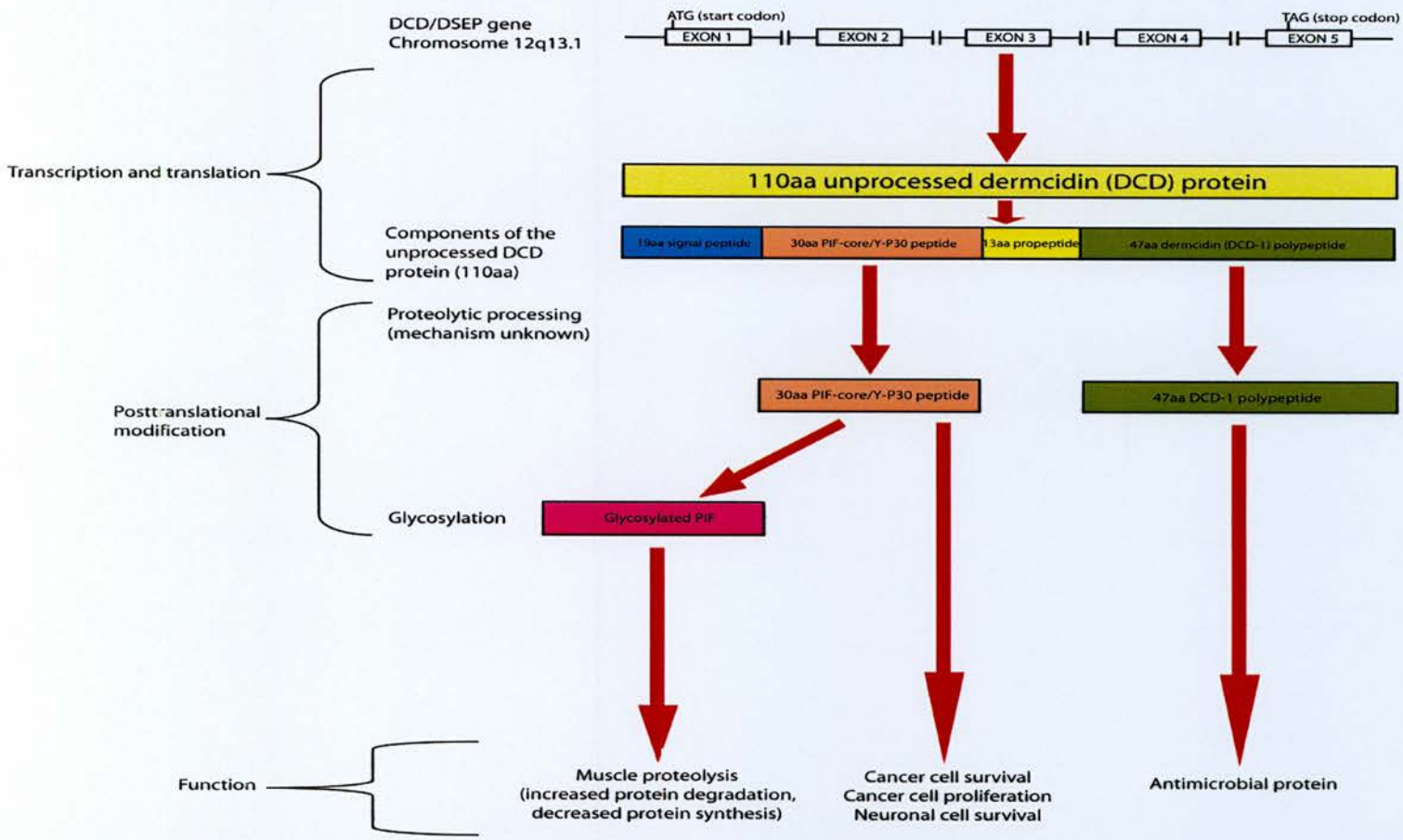

Figure 1.6 **Schematic of the *DCD* gene and the possible post-transcriptional processing of its different protein products.** The gene encoding dermcidin (known as *DCD or DSEP*) is located on chromosome 12q13.1 in humans. Its various protein products are purported to have different functions: DCD-1, an antimicrobial polypeptide; Y-P30, a cancer cell and neuronal survival peptide; and potentially glycosylated human PIF, a tumour-derived cachectic factor. The unprocessed 110-amino acid protein is also known as human cachexia associated protein (HCAP). For the DCD nucleotide and amino acid sequences, see Figure 2.1, p.181, and Lowrie *et al* [191].

was purified from medium conditioned by human retinoblastoma cells and a mouse hippocampal cell line exposed to oxidative stress [200]. When the human complementary DNA (cDNA) encoding Y-P30 was identified, the resultant native 12 kDa, 110-amino acid polypeptide (which included Y-P30 at amino acids 20-49) was named diffusible survival evasion peptide (DSEP) (Figure 1.6) [201]. Like Y-P30, DSEP was also found to increase resistance to oxidative stress. The gene encoding DSEP was identified as the same gene that encodes dermcidin [195]. Since these earlier studies, dermcidin has also been identified in human placental tissue and has been shown to have previously uncharacterised proteolytic activity [202]. Furthermore, recombinant DCD was capable of inducing an invasive phenotype in a human choriocarcinoma cell line *in vitro* [202]. Due to the absence of a reliable antibody for the detection of PIF protein in humans, in PIF studies within this thesis (Chapters 3 and 4), dermcidin mRNA levels are used as a proxy of PIF expression.

The various protein products of the *DCD* gene (Figure 1.6) are thought to depend on cleavage of the primary transcript, with or without additional post-translational modifications. However, currently there are no data regarding proteolytic processing of the DCD polypeptide into its peptide subunits. Furthermore, although many precursor polypeptides (e.g. pro-opiomelanocortin [POMC], glucagon-like peptides) undergo post-translational cleavage to form molecules with related biological activities, the discordant activities attributed to *DCD* gene products in normal and malignant cells are difficult to reconcile. The investigation of PIF is complicated even further when one considers that the murine *DCD* gene appears to

be absent from the map of the mouse genome (although the homologous gene has been described in the rat and chimpanzee).

The second PIF controversy concerns the glycosylation shell of this protein. The murine PIF-CP was reported to be extensively glycosylated, with an *N*-linked sulphated oligosaccharide chain of approximately 10 kDa, and an *O*-linked sulphated oligosaccharide chain of approximately 6 kDa [17, 203]. It is a component of this carbohydrate shell that is recognised by the existing PIF mAb upon which most PIF protein studies are reliant [17, 203]. Furthermore, *N*- and *O*-glycosylation is crucial to the muscle proteolysis induced by PIF in mice. Stable forced expression of the human gene homologue of PIF in multiple murine and human cell lines produces a non-glycosylated secreted protein that does not induce murine cachexia *in vivo* [204]. However, the absence of a site with a classical motif for *N*-glycosylation in the human PIF-CP/Y-P30 sequence remains an important barrier to proving that human cancer cells can transform the *DCD* gene product into a proteolysis-inducing factor. Sequence analysis suggests that there are 3 potential *O*-glycosylation sites and 2 potential *N*-glycosylation sites in the putative human PIF-CP. An Asp-X-Ser/Cys sequon is required for *N*-glycosylation, in which X may be any amino acid other than proline. The putative *N*-glycosylation site at asparagine residue 32 has an Asp-Pro-Cys sequence, making it possible, but unclear, if atypical N-glycosylation is likely to occur [205]. Furthermore, bioinformatic analysis indicates that the human PIF-CP is unlikely to be *O*-glycosylated at any of the putative sites in the sequence (*N*- and *O*-glycosylation were modelled using Net N-Glyc (http://www.cbs.dtu.dk/services/NetNGlyc/) and Net O-Glyc

(http://www.cbs.dtu.dk/services/NetOGlyc/), respectively) [206]. The sequences of *DCD* gene transcripts in human cell lines are analysed later in this thesis in order to detect the potential existence of any sequence variants that might increase the chances of glycosylation (Chapter 4).

The third problem surrounding PIF arises from the fact that investigations of putative glycosylated PIF in humans have all relied on a single murine mAb. The potential human homologue of PIF was reported originally to be associated specifically with the weight loss of malignant disease (based on its absence from the urine of healthy controls and weight-losing patients with trauma or sepsis) [17]. However, recent results neither confirm specificity of the presence of PIF immunoreactivity with malignancy, nor associate PIF immunoreactivity with weight loss, muscle catabolism [207, 208] or survival [183, 209]. Given these findings, an exact understanding of the specificity of the antibody is crucial. A number of recent studies have tried to use molecular tools based on the peptide portion of PIF. Polyclonal antibodies have been raised against varying fragments of the DCD protein [197, 204, 210], but none of these antibodies have been specific to the putative PIF-CP, apart from those utilised in studies in collaboration with Astra-Zeneca [191-193]. Due to steric hindrance by the two large oligosaccharide chains [203], it is not clear from the literature if an antibody to the PIF-CP would be able to bind to glycosylated PIF. Thus, in studies where antibodies to the peptide portion were used to identify and/or purify PIF from human samples, without confirmation of the presence of the proteolysis-inducing carbohydrate shell, it may be regarded that the presence of bioactive PIF was not confirmed.

Future attempts at purifying PIF may be more specific and successful following the recent characterisation of the murine PIF membrane receptor [211]. Murine PIF was found to bind with strong affinity (K_d, 1-26 nM) to sarcolemma membranes from mouse and pig skeletal muscle, murine myoblasts and a human muscle cell line [211]. Binding was competitively inhibited by chondroitin sulphate, suggesting interaction between the receptor and the sulphated oligosaccharide chains of the PIF glycoprotein. Antisera to the PIF receptor inhibited PIF-induced activation of the UPP in murine myotubes, and prevented PIF-induced weight loss in the MAC16 murine model [211]. The PIF receptor was identified as a DING protein of approximately 40kDa. Although DING proteins seem to play key roles in various human diseases, (e.g. rheumatoid arthritis, atherosclerosis, HIV), no mammalian cDNA or genomic DING sequences have been published. This is hypothesised to be due to low transcript abundance, mRNA instability, rapid turnover or gaps in the genome [212]. It has been proposed previously that DING proteins do not exist in eukaryotic cells and that any detected are due to contamination with prokaryotes, in particular *Pseudomonas* species [213]. However, the authors who characterised the receptor ensured that their cell systems were free of *Pseudomonas* [211].

Lastly, any investigation of human PIF is complicated by the fact that there are no readily available sources of glycosylated PIF. The original reports concluded that human PIF is found in very low abundance *in vivo*. Estimates suggest that PIF comprises only $5x10^{-4}$% of the total urinary protein of humans with cancer cachexia [17]. Furthermore, although the human melanoma cell line G361 has been proposed as a potential source of glycosylated PIF [198], there are no human tumour cell

lines proven to produce glycosylated PIF and thereby providing an alternative source to urine for purification. Some previous studies have relied on using recombinant, partially glycosylated PIF produced by the transfection of G361 cells with the *HCAP* (*DCD*) gene [192], but in this scenario, it is unclear as to how much such models might mimic accurately human biology.

1.6.2 Lipid mobilising factor

Zinc-α-2-glycoprotein (ZAG), otherwise known as LMF, is involved in the specific mobilisation of adipose tissue, with increased oxidation of released fatty acids, possibly via induction of uncoupling protein (UCP) expression [214]. LMF isolated from the MAC16 murine tumour, or from the urine of patents with cancer cachexia, stimulated lipolysis directly through interaction with adenylate cyclase in a guanosine triphosphate (GTP)-dependent process [16]. This effect was also produced by the interaction of LMF with the β_3-adrenoceptor [215].

Mitochondrial UCPs transport protons across the inner mitochondrial membrane, not linked to ATP synthesis. They constitute a potential energy sink and thus the overexpression of UCPs could be an aetiological factor in cachexia (although there is little evidence for this in humans). LMF has been shown to increase both mRNA and protein levels of UCP-1, -2 and -3 in brown adipose tissue (BAT) and UCP-2 in murine skeletal muscle and liver [214]. LMF is also thought to be the cause of lipid mobilisation and the decrease in plasma leptin observed in the MAC16 model. In these mice, UCP-1 mRNA levels were elevated in BAT, and UCP-2 and UCP-3 levels were increased in skeletal muscle [216]. Transgenic mice overexpressing

UCP-3 in skeletal muscle were hyperphagic and weighed less than wild type littermates, largely through a reduction in adipose tissue mass [217]. Increased levels of UCP-2 and UCP-3 mRNA expression were also demonstrated in the gastrocnemius and soleus muscles of cachectic rats bearing the Yoshida AH-130 ascites hepatoma [218]. This increase in mRNA content was associated with a two-fold increase in fatty acid, triglyceride and cholesterol levels. Reduction of hyperlipaemia with nicotinic acid also reduced UCP-3 expression in soleus, but not in gastrocnemius. This suggests that circulating fatty acids may be involved in the regulation of UCP-3 gene expression in aerobic muscles during cancer cachexia. Furthermore, a single intravenous (IV) injection of TNF-α administered to these rats caused a significant increase in skeletal muscle UCP-2 and 3 [219], suggesting that LMF and/or TNF-α may be responsible for the elevation of UCP mRNA seen in cachectic rodent muscle, possibly through elevation of serum lipid levels.

The exact cellular source of LMF is unclear from the published literature. Cachexia-inducing murine tumours (e.g. MAC16 adenocarcinoma) express ZAG mRNA at elevated levels [220], suggesting that tumour cells are the primary producers. However, other studies have demonstrated overexpression of ZAG in the white adipose tissue (WAT) of tumour-bearing mice [221], suggesting a secreted paracrine role for adipocyte-derived ZAG in the reduction of adiposity. In obese humans, expression of ZAG mRNA by adipocytes was correlated inversely with BMI and fat mass [222]. Thus, in cancer, LMF may not represent a tumour-derived cachectic mediator, but may actually be a component of the aberrant host response. The discovery of ZAG/LMF as a potential adipokine has also led to investigations

of body weight regulation in other disease conditions. Recombinant human ZAG has already shown promise as a potential treatment for Type II DM in the ob/ob mouse model [223].

Despite the apparent relationship between LMF and UCP in the regulation of body composition in animal models, evidence of a role for UCPs in human cancer cachexia is limited [224]. Some studies have been unable to demonstrate increased expression of UCPs in skeletal muscle samples from patients with cancer [225]. However, in one study, it was shown that the muscle of patients with gastric adenocarcinoma and weight loss demonstrated a five-fold increase in mRNA levels for mitochondrial UCP-3 compared with controls and cancer patients who had not lost weight [226]. If UCPs are involved in human cancer cachexia, they may offer a potential route by which LMF, a factor that causes fat catabolism, could affect indirectly skeletal muscle [227].

In the previous section, attention has focused on two specific tumour-derived cachectic mediators, namely PIF and LMF, although it has been suggested that further unidentified mediators may exist. Current controversies regarding the existence of a putative homologue of human PIF have been described, and thus it remains unclear as to whether or not PIF inhibition may be a useful anti-cachectic strategy in cancer patients. The emerging evidence of a role for LMF/ZAG in the regulation of human body composition has also been explored, but again, the functional benefits of ZAG downregulation and gain in fat mass as an anti-cachectic strategy are uncertain in cancer patients. This section represents the last

of three sections focused specifically on circulating mediators of cancer cachexia (inflammatory, neuroendocrine and tumour-derived mediators). Figure 1.7 is an expanded version of Figure 1.2 and summarises the involvement of circulating mediators within the host-tumour interaction. In the following two sections, the overall focus remains on mechanisms of cancer cachexia. However, the specific topic of discussion changes from circulating mediators of the syndrome to alternative mechanisms of weight loss and muscle wasting in cancer patients, including inadequate nutritional intake (Chapter 1.7) and mechanisms that lie outwith the bounds of the oft-considered, classical host-tumour interaction (Chapter 1.8).

1.7 Inadequate nutritional intake in cancer cachexia

Nutritional depletion is exacerbated in cancer patients by inadequate nutritional intake, which may be the result of either 'primary' anorexia and/or other secondary mechanisms (Figure 1.8).

1.7.1 Anorexia

Anorexia is defined clinically as the reduction or loss of appetite. It is experienced by approximately 15-40% of cancer patients at diagnosis, but by almost all advanced cancer patients [228]. Indeed, cancer cachexia is often termed the "cancer anorexia-cachexia syndrome", thus highlighting the significant mechanistic overlap that underlies the patient's nutritional depletion. Difficulties in clearly defining and diagnosing anorexia still exist. A VAS can be used but is unreliable if small changes in appetite are to be detected. Often, the diagnosis of anorexia is made on

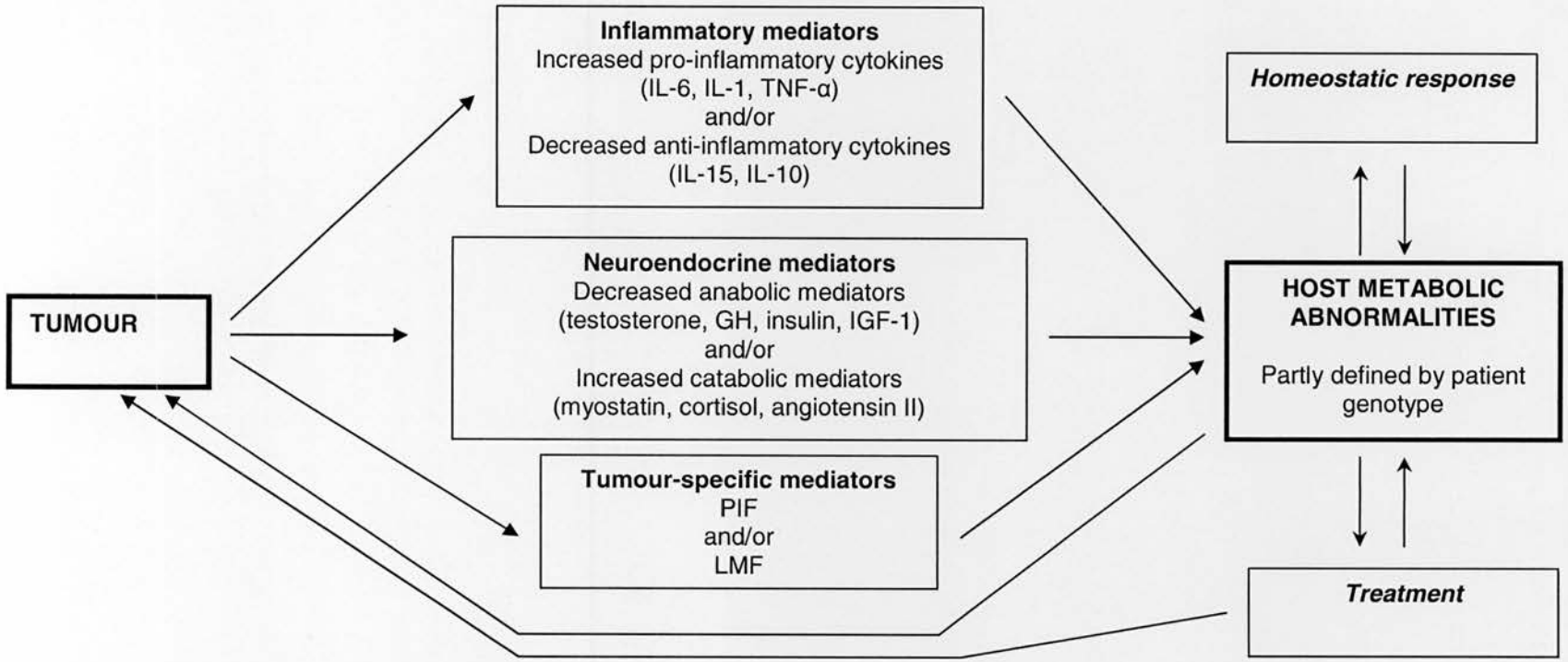

Figure 1.7 **Host-tumour interaction in cancer cachexia II.**
Different mediator pathways contribute to a variable extent within the host-tumour interaction depending, in part, on both the host and tumour type. Wasting may be the result of an excess of pro-inflammatory/catabolic mediators and/or a deficiency of anti-inflammatory/anabolic mediators. Examples of potential mediators involved are labelled on the figure. Studies of host cytokine polymorphisms have demonstrated that the host response is dictated partly by host genotype.

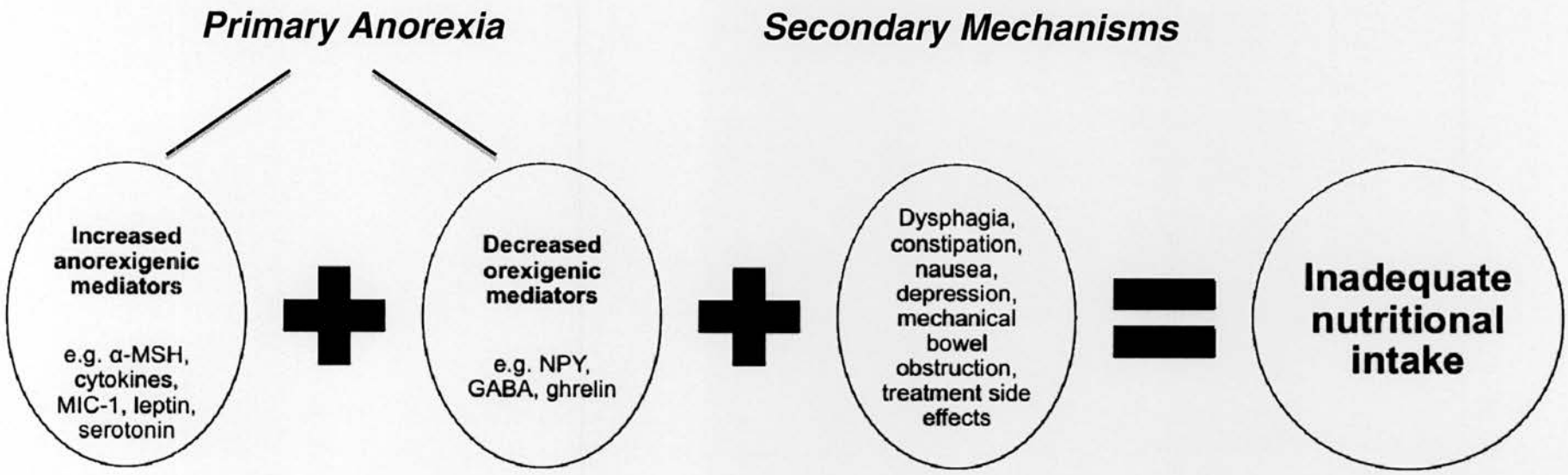

Figure 1.8 **Mechanisms of reduced nutritional intake in cancer patients.**
Inadequate nutritional intake is caused by a combination of primary anorexia (induced by certain mediators and central neuronal peptide systems) and secondary mechanisms (the result of a range of GI, affective and treatment-related symptoms). Inadequate nutritional intake will worsen the depleted nutritional status of the cancer patient by affording a diet insufficient of the protein, calories and micronutrients required for anabolism.

the basis of reduced energy intake, but this procedure can be misleading since the reduction of ingested calories might be the consequence of secondary mechanisms rather than a sign of anorexia. To assess reliably the presence of anorexia, several symptoms that interfere with food intake and are likely to be related to changes in the central control of energy intake have been identified: early satiety, taste alterations, smell alterations, meat aversion, and nausea/vomiting [229]. Patients reporting at least one of these symptoms may be defined as anorectic. However, this diagnostic tool only provides a qualitative assessment of anorexia, so it may still be advisable to quantify anorexia severity by VAS [230].

The mediators and neuronal peptide systems involved in the central regulation of appetite are discussed below. Such mediators induce either an orectic or anorectic state in the individual (Figure 1.8). However, most of the experimental work aimed at identifying these mediators has been performed in animals, and little human data are available, particularly in the context of cancer cachexia.

1.7.1.1 Neuropeptide Y and pro-opiomelanocortin systems

The pathogenesis of primary anorexia/early satiety and the control of human appetite are understood incompletely. Cytokines, (including IL-1 β, IL-6 and TNF-α), central neurotransmitters (e.g. serotonin), feeding-stimulating neuropeptides (e.g. NPY), hormones (e.g. leptin and ghrelin) and tumour-derived factors have all been implicated (Table 1.2) [230, 231]. However, two peptide systems in particular appear to be strongly influential in the control of feeding behaviour: these are the orexigenic NPY and the anorexigenic POMC systems [232]. Many mediators of

Orexigenic Mediators	Anorexigenic Mediators
Neuropeptide Y (NPY)	α-Melanocyte-stimulating hormone (α-MSH)
Gamma aminobutyric acid (GABA)	Pro-inflammatory cytokines
Ghrelin	Macrophage inhibitory cytokine-1 (MIC-1)
Agouti-related peptide (AgRP)	Leptin
Endocannabinoids	Serotonin
	Prostaglandins
	Malonyl-coenzyme A
	Parathyroid hormone-related protein (PTHrP)

Table 1.2 **Mediators of appetite control.**
Orexigenic and anorexigenic mediators that act centrally on the hypothalamus to stimulate or inhibit food intake, and are thus hypothesised to be involved in the induction of primary anorexia in cancer patients. Anorexia may be the result of an excess of anorexigenic mediators and/or a deficiency of orexigenic mediators.

appetite control exert their effects through induced changes in these two systems. Both originate in the hypothalamic arcuate nucleus (ARC) and extend projections widely over the brain [232]. They have been shown to be linked intricately with each other and to operate in parallel. POMC neurons are the source of the potent melanocortin neuropeptides, such as α-melanocyte-stimulating hormone (α-MSH), which, via interaction with the central melanocortin-3 and 4 receptors (MC3/4-R), induce an anorectic state.

1.7.1.2 Cytokines

The role of cytokines in cancer anorexia may be effected through influence on both the NPY and POMC systems. In particular, IL-1 has been associated with the induction of anorexia by blocking NPY-induced feeding [233]. NPY levels are reduced in anorectic tumour-bearing rats, and a correlation between reduced food intake and brain IL-1 was also demonstrated in the same animals [234]. The mechanism involved in the attenuation of NPY activity by cytokines may involve inhibition of NPY synthesis, inhibition of neuronal firing rates or an attenuation of its post-synaptic effects [235]. Studies in rats have also shown that the anorectic, but not pyrogenic actions, of IL-1β are modulated by central MC3/4-R [236], and that the carboxyl terminal dipeptide of α-MSH, Lys-Pro, transiently antagonises anorexia induced by IL-1β [237]. Cytokines may further mediate anorexia via an increase in corticotropin-releasing factor (CRF), a central neurotransmitter that suppresses the function of glucose-sensitive neurons and food intake. The neuroanatomical site of action of this pathway is likely to be the bed nucleus of the stria terminalis [238]. Surprisingly, in animal models, CRF-induced anorexia is not

influenced by MC-4 R blockage [239], despite the fact that α-MSH can antagonise the anorexia induced by CRF [240], suggesting the existence of a feedback loop.

1.7.1.3 Leptin

Human leptin is a protein of 167 amino acids that is manufactured in and secreted primarily by adipocytes within WAT, although a range of other tissues including skeletal muscle and gastric chief and P/D1 cells have also been shown to produce it [241]. The level of circulating leptin is directly proportional to the total amount of fat in the body [242], and in this way leptin acts as an adiposity signal. Leptin is known to increase the frequency of action potentials in POMC neurons by two different mechanisms: depolarisation through a non-specific cation channel; and reduced inhibition by local orexigenic NPY/gamma aminobutyric acid (GABA) neurons [243]. Downstream melanocortin peptides have an autoinhibitory effect on this circuit [243].

In human cancer cachexia, leptin levels are markedly low [19] (presumably because of weight loss, although other mechanisms may also be at play) and therefore melanocortin signalling should be reduced. However, studies show that the POMC system still appears to be important in the development of cancer-associated anorexia. For example, when administered into the third cerebral ventricle of Lobund-Wistar rats (an anorectic animal model of prostate cancer), the MC-R antagonist SHU9119 completely reversed anorexia and produced weight gain comparable to that observed in non-tumour bearing control animals [244]. However,

intracerebroventricular injections of NPY and ghrelin (see below, p.87) were unable to elicit the same response.

1.7.1.4 Ghrelin

Ghrelin, a 28 amino acid peptide produced by the P/D1 cells of the stomach, acts as the natural counterpart to leptin. Not only does ghrelin stimulate GH secretion (via the GH secretagogue receptor [GHS-R]-1a), but it also promotes food intake (via the orexigenic NPY system) [245] and decreases sympathetic nerve activity [246]. Ghrelin has therefore been proposed as an alternative measure to GH therapy in muscle wasting.

Patients with cancer cachexia demonstrate increased plasma concentrations of active ghrelin, suggesting a compensatory response to weight loss [247]. Intravenous ghrelin has been shown to be safe and well tolerated in patients with advanced cancer [248]. Furthermore, synthetic human ghrelin has been shown to improve muscle wasting and functional capacity in patients with cardiopulmonary-associated cachexia [249, 250], to improve energy intake in anorectic cancer patients [251], and to improve appetite and reduce loss of body fat in weight-losing cancer patients [252].

1.7.1.5 Macrophage inhibitory cytokine-1

Recently, much interest has focused on the anorectic action of macrophage inhibitory cytokine-1 (MIC-1), a tumour-secreted cytokine [253]. Studies in murine models of prostate cancer have suggested that the underlying mechanism is reduced

NPY expression and increased POMC expression in the ARC [254], and thus MIC-1 inhibition has been proposed as a potential anti-cachexia therapy in humans. However, to date, the existence of a role for MIC-1 in human cancer cachexia (either nutritional depletion or appetite suppression) has not been proven conclusively. The existence of such an association is questioned in more detail later in this thesis (Chapter 4).

1.7.1.6 Other mediators of appetite control

Other mediators of appetite control that may be over- or underexpressed during cancer-associated anorexia include serotonin, prostaglandins, malonyl-coenzyme A, endocannabinoids, and PTHrP. Changes in the circulating levels of free tryptophan induce changes in brain serotonin concentration and, consequently, changes in food intake [255]. Prostaglandins may act as mediators of anorexia in disease states associated with systemic inflammation [256]. Thus, anti-inflammatory therapies, such as cyclooxygenase (COX)-2 inhibitors, may be beneficial in the management of cancer cachexia. Malonyl-coenzyme A plays a key role in long-chain fatty acid biosynthesis. An increased level of hypothalamic malonyl CoA is an indicator of energy surplus, resulting in a decrease in food intake [257]. Endocannabinoids, lipid-derived signalling molecules (e.g. 2-arachidonoylglycerol and anandamide), are involved in the control of eating motivation via effects in the hypothalamus and nucleus accumbens [258]. Overactivity of the endocannabinoid system has been implicated in the aetiology of obesity [259]. The use of exogenous cannabinoids as an appetite stimulant for the treatment of cancer cachexia is discussed in Chapter 1.11.4.2. PTHrP has already been discussed as a pro-inflammatory mediator of

cachexia in Chapter 1.4.1.4. However, in rats, peripherally administered PTHrP has also been shown to decrease food intake and gastric emptying [64]. The decreased PA of cachectic cancer patients may also have the potential to reduce appetite by improving the sensitivity of the physiological satiety signalling system, by adjusting macronutrient preferences or food choices, and by altering the hedonic response to food [260].

1.7.2 Secondary mechanisms

Secondary mechanisms of reduced nutritional intake include physiological and psychological problems associated with the physical presence of tumour e.g. mechanical gut obstruction, dysphagia, nausea, constipation, depression, GI/oral fungal infection and side effects of treatment. Such problems should be sought actively by medical staff and appropriately managed. If not, the patient's physical status will deteriorate further, and the inanition, depression and fatigue that cachectic patients suffer will be further compounded by social isolation, as they are unable to take part in normal social patterns of eating [261, 262].

In the previous section, the high prevalence of reduced nutritional intake in advanced cancer patients has been asserted. Moreover, the underlying primary and secondary mechanisms that cause inadequate nutritional intake have been described (Figure 1.8 represents a summary diagram). The need for more research regarding the role of MIC-1 in humans has been expressed, whereas ghrelin administration has been suggested as a potential anti-cachexia therapy. In the following section,

alternative mechanisms of cachexia that lie outwith the usually considered components of the host-tumour interaction are discussed.

1.8 Beyond the host-tumour interaction

Up to this point, the present thesis has examined predominantly classical components of the host-tumour interaction, including the APPR, the neuroendocrine response and tumour-derived catabolic mediators, in the aetiology of cancer cachexia. However, there is emerging evidence that other factors including patient age, habitual levels of PA, and specifics of whole-body protein metabolism may also contribute to the deterioration of nutritional status in the cachectic cancer patient.

1.8.1 Patient age

Generally, most patients with cancer are elderly, with an average age over 70 years. Moreover, the main reason for a persistent rise in the incidence of cancer in Western society is the ever-increasing average age of the population. From the age of 50 years onwards, aging is associated with the degenerative loss of skeletal muscle, a condition known as sarcopenia [263]. The mechanisms underlying this condition are various and include reductions in circulating levels of anabolic hormones e.g. testosterone. Furthermore, sarcopenia is exacerbated by chronic illness, inadequate diet and inactivity [264]. However, one of the chief mechanisms underlying sarcopenia appears to be the anabolic resistance of elderly muscles to post-prandial amino acid loading. Normally, in the physiological post-absorptive state there is a negative balance between whole-body protein synthesis and

degradation. Any protein loss is immediately made-up in the post-prandial state by protein gain stimulated by nutrient intake [265]. In human studies, the post-prandial increase in plasma amino acid concentration stimulates muscle and whole-body protein synthesis by 50% and 40% respectively [266, 267], and whole-body protein balance is reversed from negative to positive. Sarcopenia appears to be (at least partly) the result of deficits in intracellular anabolic signalling pathways normally involved in these processes [264]. Although the basal (post-absorptive) rates of muscle protein synthesis are similar between elderly and young human muscle, elderly muscle demonstrates less anabolic sensitivity and responsiveness of protein synthesis to essential amino acids. In particular, there is an associated reduction in the expression and activation of several intramuscular mediators of protein synthesis: mammalian target of rapamycin (mTOR), p70 s6 kinase ($p70^{s6k}$), eIF4E binding protein 1 (eIF4E-BP1) and eIF2B [264] (see Chapter 1.9, p.98, for a detailed discussion of intracellular mechanisms of skeletal muscle wasting). Furthermore, there is a marked increase in expression of the inflammation associated transcription factor NF-κB [264]. The effect of insulin on leg protein breakdown is also diminished in older patients, possibly mediated by blunted Akt-protein kinase B (PKB) activity [268]. Clearly, these phenomena may not only be present in the skeletal muscle of elderly cancer patients, but may be exacerbated by the presence of ongoing systemic inflammation during cancer cachexia.

Mitochondrial dysfunction has also been proposed as part of a 'vicious cycle' theory of aging and muscle sarcopenia [269]. Enhanced ROS production, increased mitochondrial apoptotic susceptibility and reduced transcriptional drive for

mitochondrial biogenesis have all been described in sarcopenic animal models [270]. Overexpression of the mitochondrial T3 receptor induced mitochondrial activity, which resulted in sarcopenia [271]. In humans, impaired aerobic metabolism at rest and during modest levels of activity has also been observed in patients with sarcopenia [272].

1.8.2 Physical activity

In the present thesis, PF and PA are considered as different patient variables. PF is the ability to conduct a variety of everyday activities, ranging from self-care to more challenging activities requiring greater mobility, strength, or endurance, whereas PA is considered as an objective measure of physical exercise, such as the number of steps walked per day or the calorific energy expenditure spent on activity. Cancer patients may lose weight despite a normal food intake, suggesting that resting energy requirements are increased. However, in practice, measured resting energy expenditure (REE) levels have been variable. Indeed, increased [273], normal [274] and reduced REE [275] have all been described in cancer patients. It has been suggested that different tumour types (and stage of disease) may be associated with different effects on REE; lung and pancreatic cancer induced an increased REE [276, 277], whilst gastric and colorectal cancer had little effect [278]. Any increase in REE observed may be driven by an increase in mass and relative proportion of high metabolic rate tissues (such as liver and tumour) [279]. However, although REE may be increased in some hypermetabolic, wasted cancer patients, total energy expenditure (TEE) may actually fall due to a reduction in PA [277]. PA is a significant and variable component of daily energy

expenditure (EE) in free-living individuals and a reduction in PA can more than compensate for any elevation in REE. Whether such a reduction in PA represents an adaptive homeostatic response of energy regulation or a simple result of the patient's primary disease process is unclear currently. However, it seems clear that, without intervention, the cachectic cancer patient will enter a vicious circle: muscle wasting causes physical inactivity that, in turn, aggravates muscle wasting.

In a study of hypermetabolic, cachectic pancreatic cancer patients, it was shown that the measured mean physical activity level (PAL: ratio of TEE to REE) was much lower (mean 1.24) (Figure 1.9) than that recorded in healthy adults of similar age (mean 1.62) [277]. This level of PA is comparable to that observed in spinal cord injury patients living at home [280]. It is also entirely plausible that levels of activity as low as this may exacerbate wasting [281], as it is well understood that, in any individual, a lack of PA will cause deconditioning and deterioration in skeletal muscle mass. Two weeks of bed rest in healthy volunteers resulted in losses equivalent to 70g protein, or 350g wet weight of muscle [282]. Furthermore, 2 weeks of simulated microgravity (bed rest at minus-6 degrees tilt) in healthy volunteers caused a decrease in leg and whole-body lean muscle mass by 3.9% and 1.7% respectively, and a 50% reduction in muscle protein synthesis [283]. This reduction in whole-body protein with physical inactivity is a result of the loss of the stimulatory effect of PA on amino acid-mediated promotion of muscle protein synthesis [282]. The ability of combined insulin and glucose infusions to decrease whole-body proteolysis was unaffected by muscle inactivity [281], and therefore this phenomenon appears specific to amino acid-induced anabolism. Furthermore,

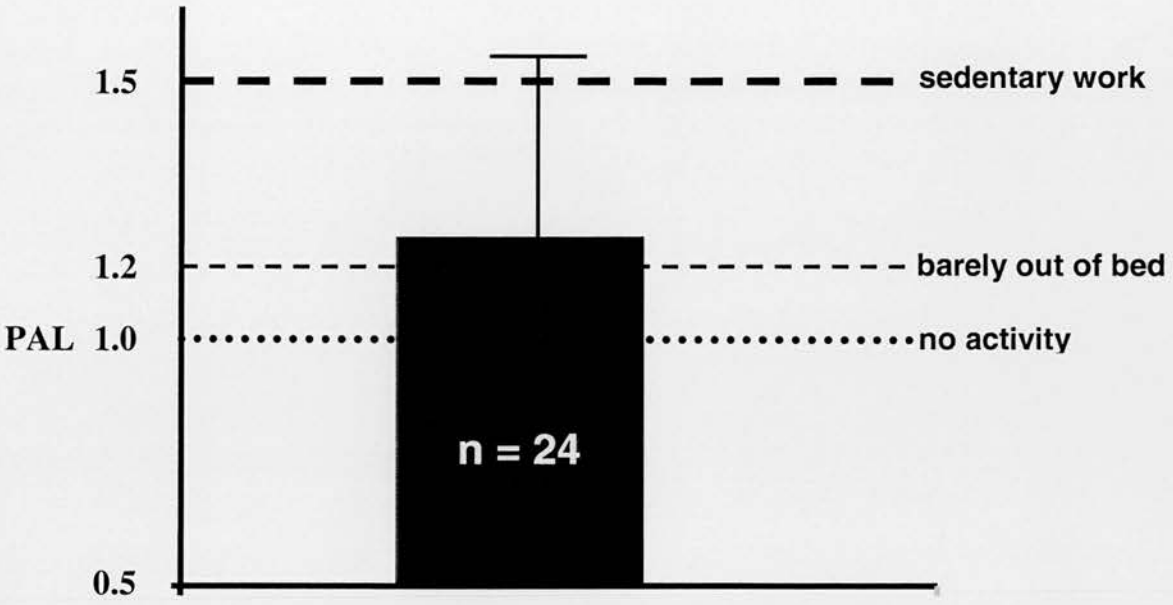

Figure 1.9 **Physical activity level of cachectic pancreatic cancer patients (n=24).**
The PAL of cachectic pancreatic cancer patients (mean 1.24) is much lower than that recorded in healthy adults of similar age. A degree of inactivity as severe as this will exacerbate skeletal muscle wasting further. Adapted from Moses *et al* [277].

the loss of anabolic stimulation by PA only tends to affect bed-rested individuals during the fed state; protein balance was similar to that observed in healthy controls during the fasted state. When combined, these results suggest that a supra-normal protein intake is required to achieve the same post-prandial anabolic effect during muscle inactivity and cachexia. It also points to the importance of maintaining even modest levels of PA in cancer.

It is worth noting that, in weight-losing pancreatic cancer patients that are not bedridden, it is possible to increase TEE and PAL with a specialised nutritional supplement containing eicospentaenoic acid (EPA) (an n-3 fatty acid with anti-inflammatory properties) administered over an 8-week period, but not with an isocaloric, isonitrogenous control supplement [277]. As PA is also an important component of the physical and social domains of QoL, any restoration of PAL towards a normal level may translate into an improvement in QoL for cachectic patients.

The topic of PA in cancer patients recurs in this introductory chapter when it will be considered as a useful outcome measure in cancer cachexia (Chapter 1.12). Moreover, the objective assessment of PA as just such an outcome measure will be investigated later in this thesis (Chapters 10 to 11).

1.8.3 Protein metabolism in the fed versus fasted state

In the past, most metabolic studies have tended to focus on patients studied in the fasted state rather than the fed state. In those studies that have investigated the post-

prandial state, feeding was associated with increased hepatic synthesis of acute phase proteins (see Chapter 1.4.2.1, p.51, and Figures 1.4 and 1.5) [73]. However, more recent investigations into the whole-body protein metabolism of fed cachectic patients have yielded intriguing results. Such studies have confirmed that whole-body protein catabolism is markedly increased in the fasting state in patients with advanced pancreatic cancer compared with healthy controls [284]. However, repeated half-hourly oral supplement feeding reduced whole-body protein breakdown rates to levels approaching those of normal controls, and interestingly, whole-body protein synthesis was increased [284]. This work emphasises the role of nutrition as a core element in the treatment of weight loss in cancer. Patients should receive small, frequent meals to attenuate protein catabolism. However, to date, no clinically applied feeding protocol has been completely successful at reversing cancer-associated weight loss.

Anti-inflammatory nutriceuticals might be the key to combining high calorie/high protein nutrition with modulation of hepatic acute phase protein synthesis. Such modulation has been demonstrated in cachectic pancreatic cancer patients receiving oral supplements enriched with EPA. After a 3-week course of treatment, not only had the patients' weight stabilised, but the combined synthetic rate of albumin and fibrinogen was reduced significantly in the fed state and demonstrated a tendency to fall in the fasting state [285].

1.8.4 Anti-neoplastic therapy

Many cancer interventions will exacerbate reduced energy and nutrient intake. Surgical patients may be fasted for prolonged periods peri-operatively, and both chemotherapy and radiotherapy can induce side effects such as anorexia, nausea, vomiting, mucositis, taste change or lethargy [286]. For example, in animal models, cisplatin-induced anorexia was mediated through reduced hypothalamic ghrelin secretion [287], whereas the ghrelin agonist, GH releasing peptide 2 (GHRP-2), reduced anorexia associated with 5-fluorouracil treatment (5-FU) in C-26 mice [288]. Symptoms will depend on the nature and course of the chemotherapeutic drugs being utilised, and the location, volume and dose of radiotherapy.

Some cytotoxic drugs may even generate their own cachexia-like side effects. For example, antitubulin taxanes induced greater loss of body weight in tumour–bearing mice than in healthy mice, even when the agents significantly reduced tumour growth [289]. Moreover, CT analysis of patients with metastatic renal cell cancer resistant to standard therapy demonstrated that individuals receiving sorafenib lost skeletal muscle progressively at 6 months (decrease of 4.9%) and 12 months (decrease of 8.0%), whereas patients receiving placebo were weight-stable [290]. The complex interaction between nutrition, cachexia and chemotherapy still requires further elucidation [291, 292].

In the previous section, mechanisms of skeletal muscle wasting outwith the usually considered components of the host-tumour interaction, including elderly patient age, physical inactivity, treatment-related side effects, and specific aberrations in protein

metabolism demonstrated by cancer patients, have all been discussed (Figure 1.10 represents a summary diagram). The treating physician should appreciate such 'non- classical' mechanisms, as immediate recognition of these additive causes of nutritional depletion may reduce the time to early prophylactic intervention. The following section is the last addressed specifically to mechanisms of cancer cachexia, and the thesis now focuses on intracellular mechanisms of skeletal muscle proteolysis.

1.9 Intracellular mechanisms of skeletal muscle wasting

Muscle protein is continually synthesised and broken down each day with an average turnover rate in humans of 1-2% [293]. Under normal circumstances, synthesis equals breakdown and muscle bulk remains constant. Muscle wasting occurs when synthesis is decreased, breakdown is increased, or a combination of events leads to a net negative balance. In cancer cachexia, the initial loss of muscle may appear subtle but, over prolonged periods of time, severe wasting with >75% loss of skeletal muscle mass can occur [1].

Although the major focus of this thesis is skeletal muscle wasting in cancer cachexia, much of the important work that has led to our understanding of the underlying molecular mechanisms has been performed in other models of wasting, and so these models are also discussed below. Indeed, the mechanistic similarities between cancer cachexia and muscular dystrophy are studied in more detail later in this thesis (Chapter 7). However, as with the data regarding anorexia, most of the

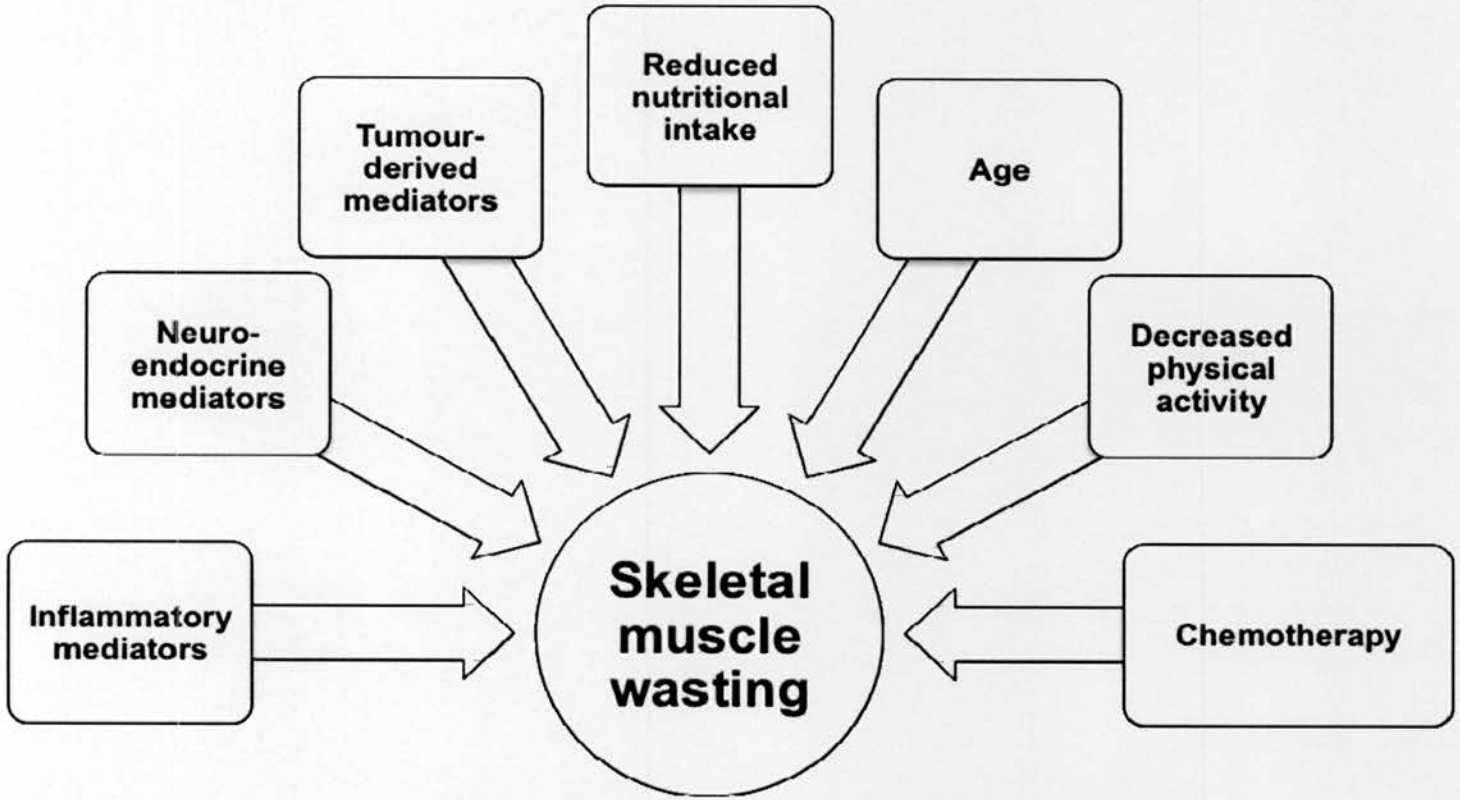

Figure 1.10 **Factors that induce skeletal muscle wasting in cachectic cancer patients.**
Circulating cachectic mediators, reduced nutritional intake, patient factors (including advancing age and physical inactivity), and treatment-related side effects can all induce skeletal muscle wasting.

experimental work has been performed in pre-clinical models, rather than human patients.

1.9.1 Intracellular signalling pathways in skeletal muscle wasting

Several transcription factors and intracellular signalling pathways have been implicated in the regulation of skeletal muscle wasting. These pathways may be largely anabolic or catabolic in nature, but there is likely to be significant crosstalk between the individual cascades. Muscle wasting results from a chronic imbalance in the activation of these different pathways. The major catabolic pathways involve cytokine-induced activation of NF-κB signalling, and the upregulation of members of the proteolytic UPP via NF-κB or the Forkhead box, class O (FOXO) transcription factors (Figure 1.11). Alternatively, myostatin, a major inhibitory muscle GF, can prevent muscle differentiation and repair by inhibiting the synthesis and activity of MyoD, an essential transcriptional regulator of these activities (Figure 1.12). The major anabolic pathway is the IGF-1/PI3K/Akt pathway that results in the phosphorylation of mTOR and $p70^{s6k}$, and the subsequent increase in protein synthesis (Figure 1.11).

1.9.1.1 *Nuclear Factor-Kappa B*

In recent years, a vital role has emerged for NF-κB signalling in the control of muscle degradation. There are five known mammalian NF-κB transcription factors (p65 [Rel A], Rel B, c-Rel, p52 and p50), and all are expressed in skeletal muscle. Together they modulate a variety of processes, including apoptosis, inflammation and differentiation, that are dependent on cell type and upstream triggers. TNF-α is

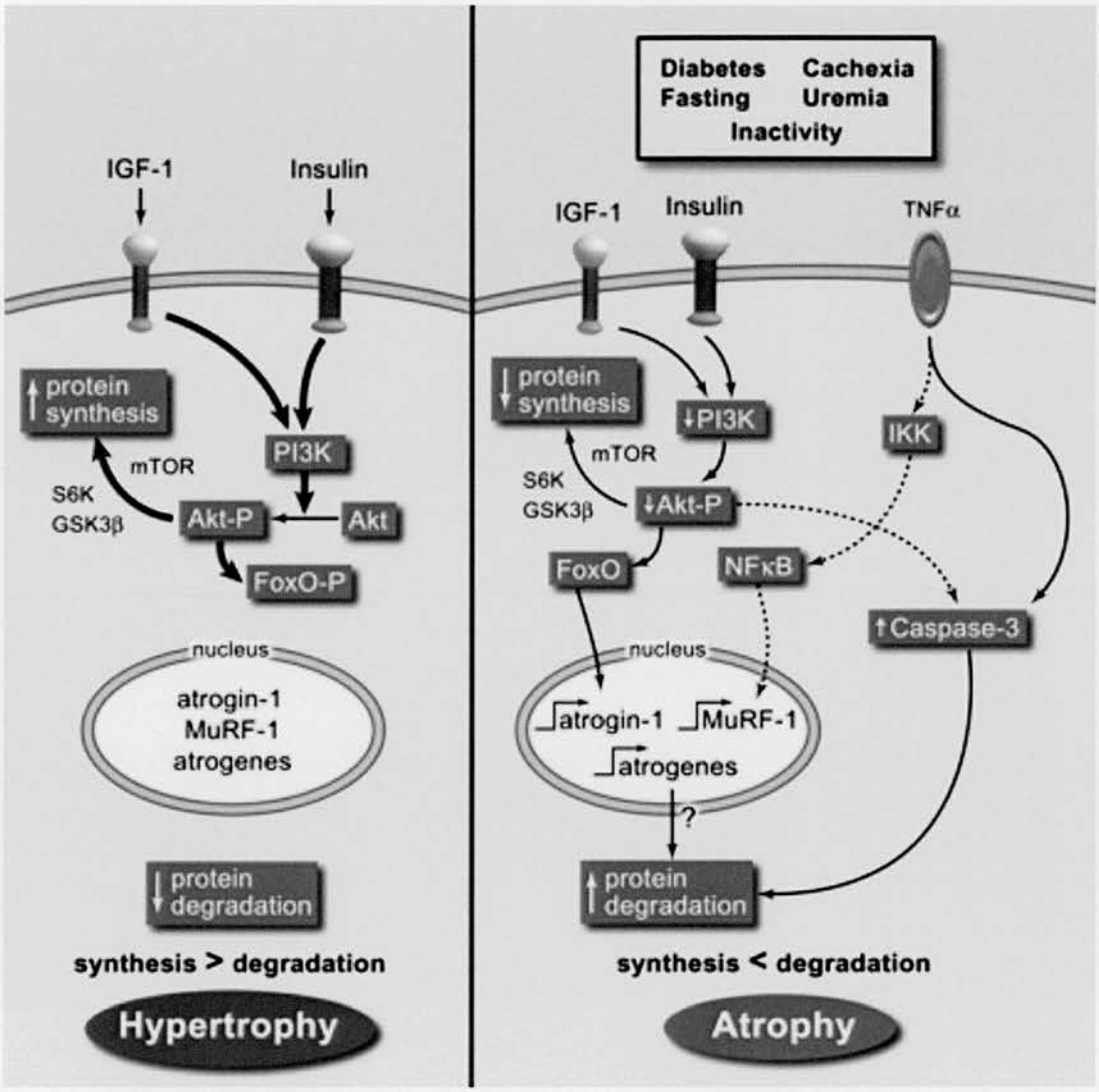

Figure 1.11 **The major catabolic and anabolic signalling pathways involved in skeletal muscle homeostasis.**
The balance between protein synthesis and degradation determines whether muscles hypertrophy or atrophy. In diseases states that induce muscle atrophy, insulin/IGF-1 signalling is suppressed and the PI3K/Akt pathway is depressed, leading to decreased protein synthesis and FOXO phosphorylation. This allows FOXO to stimulate the expression of the E3 ubiquitin ligases MAFbx/atrogin-1 and MuRF-1 (see later, p.105), leading to protein degradation. Inflammatory cytokines, such as TNF-α, also signal through the IKK complex, which causes IκBα phosphorylation and degradation, and subsequent NF-κB nuclear translocation, leading to transcriptional regulation of MuRF-1 and proteolysis. From Lecker *et al,* 2006 [164].

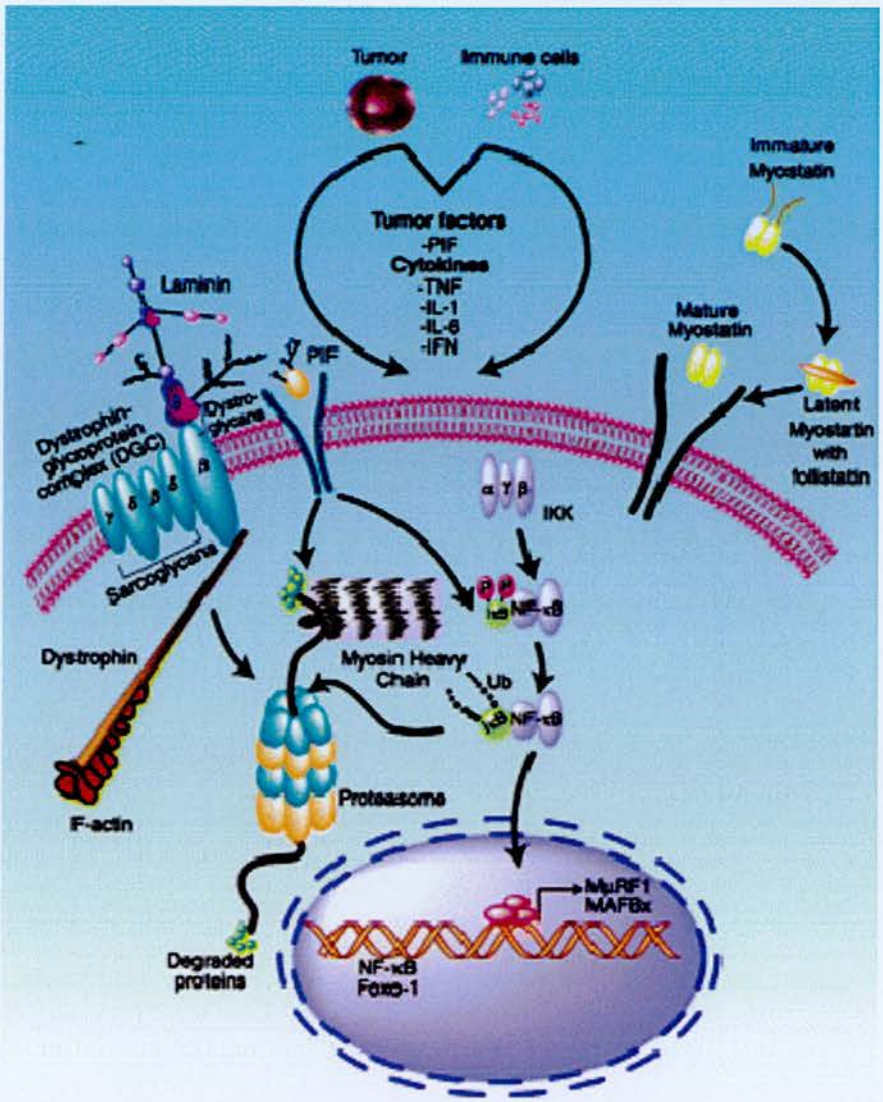

Figure 1.12 **Emerging intracellular signalling pathways of muscle wasting in cancer cachexia.**
Myostatin in its cleaved and active form, signals through a type II receptor to regulate muscle atrophy through the induction of Foxo-1 and MAFBx/atrogin-1, a process that is independent of NF-κB. Also contributing to this signalling is the newly implicated dystrophin glycoprotein complex, whose dysfunction by an unknown mechanism regulates muscle wasting through the induction of MuRF1 and MAFBx/atrogin-1. Shown in the cytoplasm is the MyHC protein that is a selective myofibrillar protein target of the proteasome system in cancer cachexia. From Acharyya and Guttridge, 2007 [294].

the classical activator of NF-κB in a variety of cell types, and NF-κB is necessary for TNF-induced muscle atrophy [295]. TNF-α induces the biphasic cytosolic release of NF-κB from its inhibitory IκB proteins to allow translocation of NF-κB into the nucleus and subsequent transcription of proteolytic pathway components [295]. In both myocytes and murine models, the cytokine-induced activation of NF-κB has been shown to inhibit muscle differentiation and function through the suppression of MyoD mRNA and protein [296]. In fact, chronic muscle-specific NF-κB activation in transgenic mice is associated with profound muscle wasting due to accelerated ubiquitin-dependent proteolysis [297]. In contrast, *NF-κB* knockout mice, specific to the p50 subunit, do not demonstrate the usual decrease in muscle fibre size associated with a period of unloading [298]. However, even in non-inflammatory models of muscle wasting that do not involve cytokine activation (e.g. disuse atrophy), the activation of NF-κB through non-classical pathways has been demonstrated. In animal models of disuse atrophy, Bcl-3, a transcriptional co-activator and singular member of the IκB family, is upregulated [298]. Importantly, mice null for *Bcl-3* demonstrate complete inhibition of unloading-induced NF-κB activity [298].

Regarding cachexia, daily treatment of rats bearing the Yoshida AH-130 ascites hepatoma with a double inhibitor of NF-κB and activator protein-1 (AP-1) caused a significant recovery of muscle weight [299], whereas skeletal muscle from cachectic COPD patients demonstrated a 30% increase in NF-κB DNA-binding activity [300].

1.9.1.2 IGF-1/PI3K/Akt pathway

The PI3K pathway is an anabolic pathway that demonstrates robust experimental evidence of chronic deactivation in muscle wasting [301]. During anabolism, activation of PI3K pathway by upstream ligands, such as insulin and IGF-1, leads to activation of Akt (a serine-threonine kinase), which in turn phosphorylates and activates the mTOR kinase. The activation of mTOR then results in increased protein synthesis via the phosphorylation and activation of $p70^{s6k}$, and phosphorylation of eIF4E-BP1, which relieves the repression of eIF4E. In several different models of wasting, a marked decrease in the phosphorylation of Akt, mTOR and $p70^{s6k}$ has been demonstrated [302, 303]. Furthermore, transgenic mice with disruption of the *Akt1* gene demonstrate growth retardation, whereas mice with knockout of both the *Akt1* and *Akt2* genes exhibit significant muscle atrophy [304].

Interestingly, both the NF-κB and PI3K pathways are capable of influencing each other. For example, Akt stimulates NF-κB-dependent transcription by stimulating the transactivation domain 1 of the p65 subunit (in an IκB kinase [IKK]-dependent pathway) [305]. In contrast, oncogenic Ras, an upstream activator of Akt, requires NF-κB to initiate transformation [306].

In muscle samples from cachectic patients with pancreatic cancer, Akt protein level was decreased by 55%, and the phosphorylated forms of mTOR and $p70^{s6k}$ were decreased by 82% and 39%, respectively, compared with non-cachectic individuals [307].

Alterations in the expression levels of intracellular pathways involved in human muscle protein translation and synthesis during cancer cachexia are studied in more detail later in this thesis (Chapter 8). In particular, the roles of PKR, an enzyme activated by double-stranded RNA (dsRNA) produced in response to viral infection, and its substrate, eIF2α, are explored in muscle samples from cachectic patients.

1.9.1.3 Forkhead Box, Class O transcription factors

The FOXO family of gene transcription factors has been shown to regulate expression of vital components of the proteolytic UPP, namely the E3 ligases muscle-specific F-box (MAFbx) (also known as atrogin-1) and muscle-specific RING ('Really Interesting New Gene') finger-1 (MURF-1) [308] (see Chapter 1.9.2.4, p.116), in various models of skeletal muscle wasting, including cancer cachexia, DM, fasting, aging, angiotensin II-induced, and dexamethasone-induced [309]. These transcription factors are under inhibitory control by the IGF/Akt/PI3K pathway [310]. When Akt is activated, FOXO is phosphorylated and bound by 14-3-3 proteins that mediate its movement from the cell nucleus to the cytoplasm. Inactivation of Akt leads to dephosphorylation and nuclear localisation of FOXO, with subsequent activation of genes involved with cell death, cell cycle inhibition and metabolism. In particular, FOXO-3 upregulates atrogin-1, resulting in decreased muscle fibre size, while FOXO-1 is induced specifically in atrophying muscle [309].

Reduced levels of phosphorylated (inactive) FOXO3a have been observed in the skeletal muscle of cachectic pancreatic cancer patients compared with non-

cachectic individuals, but an unexplained twofold reduction in the total amount of FOXO1 and FOXO3a was also seen [307].

19.1.4 Dystrophin glycoprotein complex

Cancer cachexia represents an acquired syndrome of skeletal muscle wasting, but a separate group of muscle disorders also characterised by severe muscle wasting are the muscular dystrophies. Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD) and several forms of limb-girdle muscular dystrophy (LGMD) have been linked to mutations in different genes that encode components of the dystrophin glycoprotein complex (DGC), a multi-protein structure associated with myofibre membranes (Figure 1.13) [311-313]. At the core of the DGC is dystrophin, a large 427kDa protein that associates with the cytoskeleton through its interactions with F-actin at its amino terminus and connects to the sarcolemma by binding to β-dystroglycan (β-DG) at its carboxyl end [314-316]. Dystroglycan is made of α and β forms, which are derived from a single protein precursor through post-translational processing. β-DG is tightly bound to α-DG, which itself is linked to the ECM by its interactions with laminin-2. The DGC therefore forms a strong mechanical link between the cytoskeleton and the ECM, protecting cells from contraction-induced injuries [317]. In addition, the DGC maintains an active signal transduction pathway by interacting with growth factor receptor-bound protein 2 (Grb2) and neuronal nitric oxide synthase (nNOS) [318]. Mutations in dystrophin or other members of the DGC disrupt the mechanical linkage and/or signalling pathway(s) resulting in membrane damage, necrosis, and eventual muscle wasting [320].

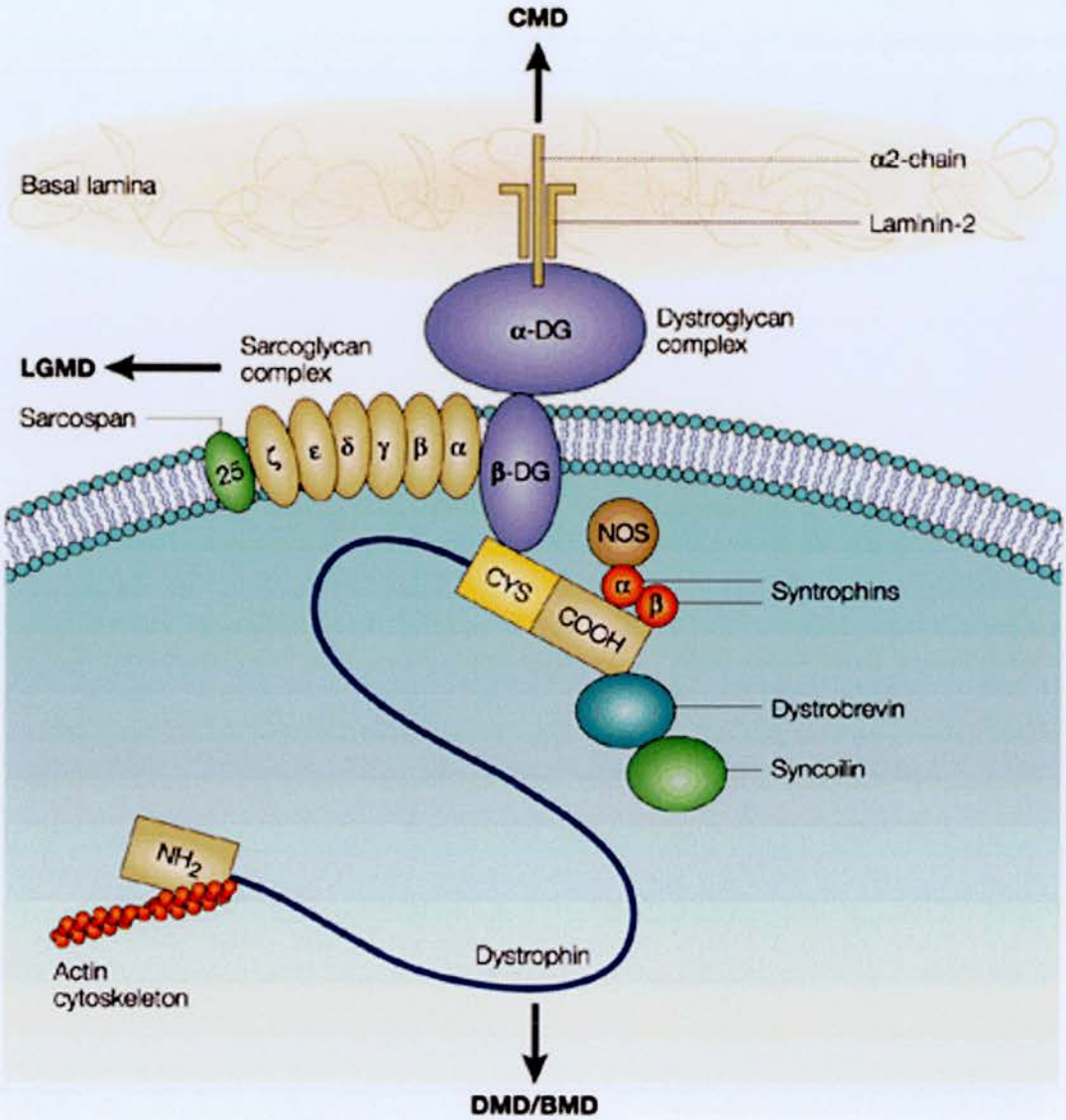

Figure 1.13 **The dystrophin–glycoprotein complex and aetiology of the muscular dystrophies.**
The DGC provides a strong mechanical link between the cytoskeleton and the extracellular matrix protecting cells from contraction-induced injuries. Mutations in dystrophin and other DGC members cause a variety of muscular dystrophies. Adapted from Khurana and Davies, 2003 [319].
BMD, Becker muscular dystrophy; CMD, congenital muscular dystrophy; CYS, cysteine; DG, dystroglycan; DMD, Duchenne muscular dystrophy; LGMD, Limb girdle muscular dystrophy; NOS, nitric oxide synthase.

In recent years, it has been demonstrated that alterations in the muscular dystrophy-associated DGC also represent a key initiating event in skeletal muscle wasting in animal models of cancer cachexia (Figure 1.12) [321]. Using both the C-26 and Lewis lung carcinoma (LLC) murine cancer models, it was shown that muscles from tumour-bearing mice exhibited membrane abnormalities, accompanied by reduced dystrophin (core DGC member) expression and increased glycosylation of DGC proteins [321]. However, the existence of a role for DGC deregulation in skeletal muscle wasting within human cancer cachexia has not been proven. This question is addressed in detail later in this thesis (Chapter 7).

1.9.2 Intracellular effector mechanisms of skeletal muscle wasting

Several different intracellular effector mechanisms of muscle proteolysis exist. The system believed to degrade the major contractile skeletal muscle proteins (monomeric actin and myosin) is the UPP. However, the UPP cannot break down actomyosin complexes or myofibrils, and therefore other proteolytic enzymes are required to act upstream (m-calpain, cathepsin-L and/or caspase-3) and downstream (tripeptidyl peptidase II and aminopeptidases) of the UPP for the complete degradation of myofibrillar proteins into free amino acids.

1.9.2.1 Caspases

Caspases (**cys**teine-**asp**artic acid prote**ases**) are a group of enzymes usually associated with the induction of cellular apoptosis in response to death signals. There are two types of caspases: initiator caspases and effector caspases. Initiator caspases (e.g. caspase-8, caspase-9) cleave inactive pro-forms of effector caspases,

thereby activating them; effector caspases (e.g. caspase-3, caspase-7) then cleave other protein substrates within the cell, resulting in apoptosis. This process may be important in muscle wasting during the apoptotic response to calcium pathways and TNF-α (which can activate caspase-8 and -10) [322]. However, perhaps the most significant action of caspases in muscle wasting is during the initial steps of myofibrillar degradation. Increased caspase-3 activity associated with increased actin fragmentation has been shown in experimental muscle wasting associated with both DM and angiotensin II [323].

Caspases, along with PW1/Peg3 and bax, are usually considered as downstream effectors of the p53 cell death pathway. TNF-α has been shown to inhibit myogenic differentiation and skeletal muscle regeneration *in vitro* via PW1/Peg3, bax, and caspases [324]. Furthermore, the p53 pathway was implicated in muscle wasting experienced by mice inoculated with the C26 tumour [325]. Gene transfer of a dominant-negative form of PW1 into murine muscle tissue *in vivo* also blocked myofibre atrophy in response to tumour load [325]. Taken together, these results may point to a novel role for p53 in mediating muscle wasting during cancer cachexia.

There is some evidence of a role for apoptosis in muscle wasting experienced by cachectic human patients. Muscle biopsies from weight-losing patients with upper GI cancer showed a three-fold increase in muscle DNA fragmentation compared with control subjects [326]. Furthermore, the increase in DNA fragmentation was associated with a four-fold increase in poly (adenosine diphosphate-ribose)

polymerase (PARP) cleavage, indicating the presence of apoptosis [326]. Apoptosis has also been identified in the muscles of patients with CHF and is associated with impaired exercise tolerance [327]. However, these apoptotic changes were not specific to patients with cachexia. Also, circulating levels of soluble Fas ligand, one of the key initiators of the death pathway, are higher in cachectic COPD patients compared with non-cachectic patients, but levels are lower than those observed in healthy controls [328], implying that the overall regulation of muscle apoptosis in human disease states requires further elucidation.

1.9.2.2 Calcium-dependent enzyme systems

Several different calcium-dependent enzyme systems exist, including the calpains and the Ca^{2+}/calmodulin-dependent protein kinases (CaMK). The calpains are a family of intracellular, non-lysosomal, calcium-regulated cysteine proteases that mediate cleavage of specific substrates in a large number of regulatory cell processes [329]. The calpain family includes three ubiquitous enzymes and several tissue-specific forms.

In excessive amounts, calpains are capable of degrading cytoskeletal elements, ion channels, other enzymes, cellular adhesion molecules and cell surface receptors. Furthermore, calpains cleave the enzyme xanthine dehydrogenase to xanthine oxidase (XOA), thus indirectly creating the formation of superoxide radicals. This pathway is upregulated in CHF, and radical-induced impairment of NO-dependent vasodilator capacity is a characteristic feature of CHF pathology [330].

Hyperuricaemia is thus a strong and independent marker of impaired prognosis in CHF [331].

Muscle tissue expresses three different calpains: 1, 2 and 3. The ubiquitous calpains 1 and 2 (also called μ and m) have been heavily implicated in both the initial degradation of myofibrillar proteins during muscle wasting [332] and the necrosis associated with muscular dystrophy [333]. Ubiquitous calpain activity is under the control of the endogenous inhibitor calpastatin, and downregulation of calpastatin expression has been suggested as one potential mechanism of muscle atrophy. In contrast, overexpression of calpastatin may have potential therapeutic benefits against muscle atrophy. In animal models, transgenic overexpression of calpastatin can reduce both the atrophy caused by unloading and the necrosis associated with muscular dystrophy [334].

The role of muscle-specific calpain 3 is less well understood, but experimental results would suggest that calpain 3 inhibition is required for atrophy to occur. For example, calpain 3 expression is often downregulated in wasting models [335], whereas inactivating mutations in the *calpain 3* gene are responsible for limb-girdle muscular dystrophy type 2A [333]. Studies performed on muscle biopsies from patients with this latter condition have led to the postulation that the normal function of calpain 3 is to intervene in the regulation of expression of NF-κB-dependent survival genes to prevent skeletal muscle apoptosis [336]. Thus, calpainopathies lead to deregulation of NF-κB activity and resultant muscle wasting.

Although calpain 3 expression has not been analysed in muscle samples form cachectic cancer patients, increased muscle mRNA levels for calpain 2 and cathepsin D (see Chapter 1.9.2.3, p.113) have been detected in critically ill ICU patients with severe head trauma [337].

Recent mRNA and protein studies have demonstrated that expression of CaMKIIß, a serine/threonine-specific protein kinase regulated primarily by the Ca^{2+}/calmodulin complex, is elevated in muscle samples from cachectic patients with upper GI cancer [338]. CaMKIIβ protein levels and activity normally increase after exercise training [339], implying that cancer cachexia does not simply reflect a profile of physical inactivity. It has also been suggested recently that Ca^{2+}-CaM-eukaryotic elongation factor-2 kinase (eEF2K) signalling may be responsible for acute exercise-induced inhibition of muscle protein synthesis [340], and it is thus conceivable that chronic inappropriate activation of this 'endurance training'-related signalling molecule [341] subdues normal maintenance of skeletal muscle mass. Additional factors that could modulate CaMKII activity in cachectic cancer patients include alterations in lipid metabolism [342] and generation of ROS. CaMKII has also been implicated in muscle adaptation through phosphorylation of histone deacetylase 5 (HDAC5) leading to MyoD/myocyte enhancer factor-2 (MEF2)-driven differentiation of muscle cells [343]. Therefore, it is plausible that CaMKII activation within cachectic muscle is a compensatory strategy in the face of failing protein synthesis. Alternatively, the CaMKIIβ response may simply indicate failure of calcium homeostasis, a factor that would also activate proteolytic calpain and caspase activity [344, 345].

1.9.2.3 *Lysosomal enzymes and autophagy*

Intracellular lysosomes contain a number of enzymes required for the digestion of macromolecules, including proteases, carbohydrases, lipases and nucleases. Cathepsins L, B, D and H are the major lysosomal proteases and determine primarily the proteolytic capacity of lysosomes. Although represented poorly in adult skeletal muscle compared to foetal, lysosomal proteases are upregulated in pathological conditions [346]. Cathepsin-L, in particular, is induced early in catabolic states, and levels correlate with increased protein breakdown. Increased cathepsin activity has been demonstrated in various models of muscle wasting, including cancer cachexia, DM, dexamethasone-induced, disuse, fasting and muscular dystrophy [347, 348].

Lysosomes may also induce muscle wasting by taking part in autophagy, the absorption and digestion of cytoplasm and intracellular organelles, which is required for cellular survival and for the clearance of damaged proteins and altered organelles. Autophagy of skeletal muscle is observed commonly in response to starvation, denervation, ageing and oxidative stress [349].

In most mechanistic studies, induction of autophagy has been associated with increased activation of FOXO3A-dependent transcription of autophagy-related genes such as *LC3B* and *BNIP3* [350]. In particular, BNIP3 induces autophagosome formation and is responsible primarily for the induction of autophagy by FOXO3A. Rapamycin is not able to induce autophagy in skeletal muscle *in vivo*, thus indicating that it is the Akt-FOXO axis that is involved in autophagy, rather than

the Akt-mTOR pathway [350]. Alternative mediators/mechanisms of autophagy may also include increased activation of FOXO1 [351], increased activation of p38 mitogen-activated protein kinase (MAPK) [352], and reduced levels of superoxide dismutase (SOD) [353].

Autophagy has been proposed as a potential link between electrical activity (in the form of excitation-contraction coupling) and muscle wasting. It has been suggested that the T-tubule (deep invagination of the sarcolemma which transmits depolarisation to the interior of the cell) is the membrane donor that forms sequestering autophagic vesicles [353]. Furthermore, L-type Ca^{2+} channels have also been offered as voltage-gated sensors that are linked to transcriptional activity controlling muscle differentiation [354]. Downregulation of the dihydropyridine receptor (DHPR) α1S subunit (which forms the L-type Ca^{2+} channel in T-tubules and is also the voltage sensor of excitation-contraction coupling at the level of triads) caused severe muscle atrophy in animal models [354]. Moreover, decreased levels of Runx1 (AML1), a DNA-binding protein that has critical roles in haematopoiesis and leukaemogenesis (AML represents 'Acute Myeloid Leukaemia'), and that regulates transcription of genes encoding ion channels, signalling molecules and muscle structural proteins, have been implicated as another potential causative factor in autophagy-associated wasting [355].

In humans, data indicating a role for autophagy in muscle wasting are limited currently. However, a couple of supportive studies do exist. In patients receiving controlled mechanical ventilation, autophagy of the diaphragmatic muscle has been

detected [356]. In latissimus dorsi samples from resectional lung cancer patients, cathepsin B mRNA levels were elevated compared with controls, and correlated with fat-free mass (FFM) index and tumour stage [357]. Furthermore, in patients with cancer cachexia, modestly increased transcription of the autophagy-related genes *GABRAPL1* (*GABA receptor-associated protein-like 1*) and *BNIP3* was demonstrated in rectus abdominis compared with weight-stable cancer patients and controls [338]. However, as autophagy also plays a normal role in muscle cell homeostasis and survival, repression of autophagy as a potential therapeutic strategy in cancer cachexia may not represent a viable tactic. Inducible, muscle-specific knockout of the gene *ATG7* (which is critical for autophagy) in mice revealed an unexpected phenotype characterised by muscle atrophy, weakness and features of myofibre degeneration, including presence of protein aggregates, abnormal mitochondria, accumulation of membrane bodies, sarcoplasmic reticulum distension, vacuolisation, oxidative stress and apoptosis [358]. Moreover, autophagy inhibition did not protect skeletal muscles from wasting during denervation and fasting, but instead promoted greater muscle atrophy. Furthermore, in humans, X-linked myopathy with excessive autophagy (XMEA) is a childhood-onset disease characterised by progressive atrophy of skeletal muscle, which appears to be the result of reduced lysosomal degradative ability [359]. XMEA is caused by hypomorphic alleles of the *VMA21* gene, which encodes for an essential assembly chaperone of the major mammalian proton pump complex, vacuolar-type H^+-ATPase (V-ATPase; ATP is adenosine-5'-triphosphate) [359]. Decreased VMA21 raises lysosomal pH, which leads ultimately to macroautophagic overcompensation and cell vacuolisation.

1.9.2.4 *Ubiquitin-proteasome pathway and E3 ligases*

Most models of muscle wasting have suggested that the dominant mechanism of proteolysis is increased activity of the UPP (Figure 1.14). In addition to degrading misfolded proteins, the UPP is involved normally in the destruction of proteins that are under temporal (e.g. cell cycle regulators) or environmental (e.g. several transcription factors) control. The UPP is therefore essential for the regulation of cell cycling, signal transduction and gene expression [164].

In the UPP, target proteins are identified for degradation by being covalently bonded with a polyubiquitin chain (Figure 1.14). This process of ubiquitination is under the control of an E3 ubiquitin ligase, which operates in conjunction with an E1 ubiquitin-activating enzyme and an E2 ubiquitin-conjugating enzyme [164]. The E3, which may be a multi-protein complex, is generally responsible for the selection and specific timing of targeting ubiquitination to specific substrate proteins. There are several families of E3 ubiquitin ligases that are characterised by particular protein domains. Following polyubiquitination, target proteins are recognised by the 26S proteasome, a large ATP-dependent multicatalytic protease, which removes the ubiquitin chain and degrades the proteins into 8-11 amino acid oligopeptides. The 26S proteasome spans the cell's nuclear membrane and consists of a 20S core protease particle and two 19S regulatory particles.

Increased mRNA and protein expression of components of the UPP have been demonstrated in several models of muscle atrophy, including those induced by cancer cachexia [360], glucocorticoids, and angiotensin II [164]. Recently, two

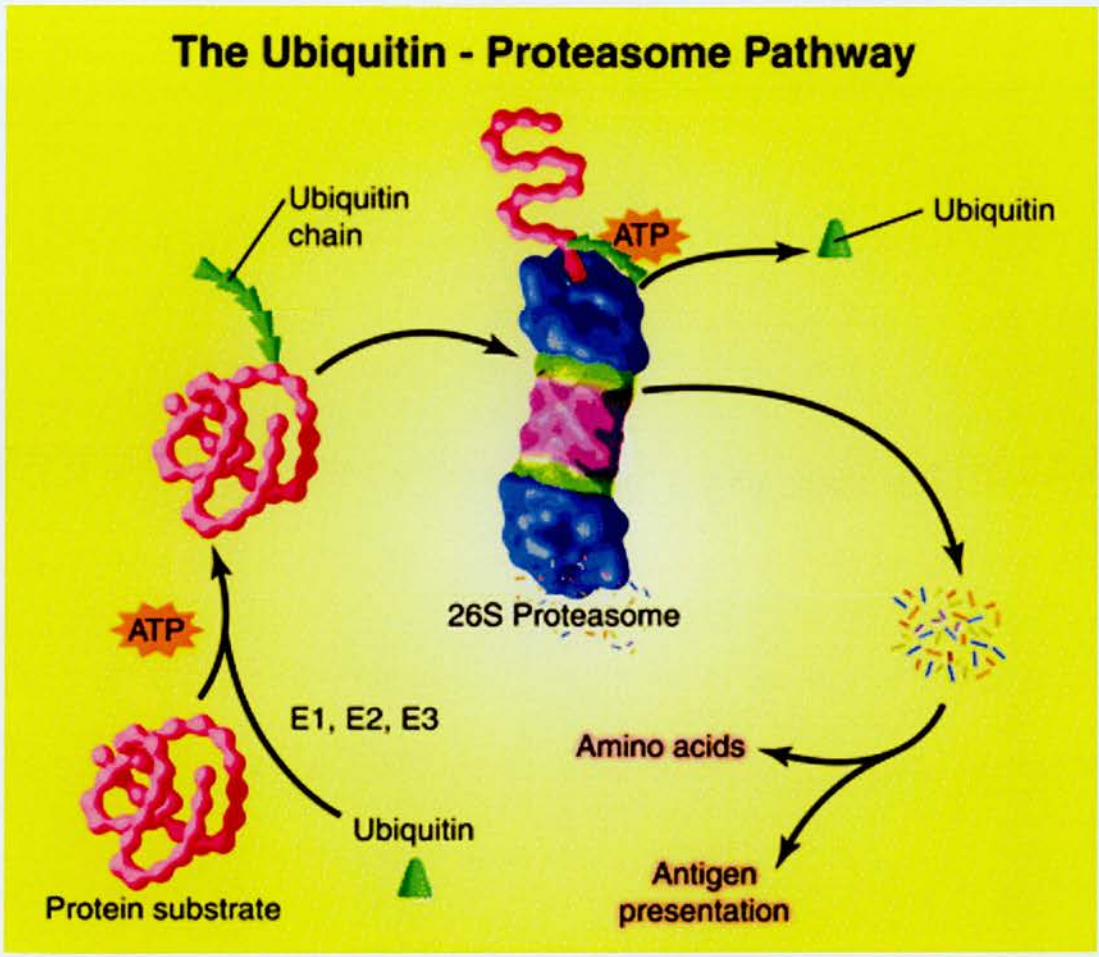

Figure 1.14 **The ubiquitin-proteasome pathway of protein degradation.** The ubiquitin-proteasome pathway is a complex multi-step, ATP-dependent process, which requires 3 enzymes and which results in the tagging of specific proteins with ubiquitin for degradation in the proteasome. A chain of 5 ubiquitin molecules attached to the protein substrate is sufficient for the complex to be recognised by the 26S proteasome. In addition to ATP-dependent reactions, ubiquitin is removed and the protein is linearised and injected into the central core of the proteasome, where it is digested to peptides. The peptides are then degraded to free amino acids by peptidases in the cytoplasm or used in antigen presentation. From Lecker *et al,* 2006 [164].
E1 = ubiquitin-activating enzyme; E2 = ubiquitin-conjugating enzyme; E3 = ubiquitin ligase.

muscle-specific E3 ligases, MAFbx and MURF-1, have been identified that seem to be of profound importance in a wide range of atrophies. MAFbx and MURF-1 knockout mice demonstrated 56% and 36% attenuation in gastrocnemius wasting, respectively, following 14 days of denervation [308].

Other ubiquitin ligases may also be important in skeletal muscle protein degradation via regulation of the 'N-end rule' pathway, a mechanism that relates the *in vivo* half-life of a protein to the identity of its N-terminal residue [361]. E3α-II, a novel N-end rule ubiquitin ligase, has shown to be highly expressed in the skeletal muscle of both the Yoshida hepatoma rat cachexia model and the C-26 mouse model, accompanied by a dramatic increase in protein ubiquitination [362]. Furthermore, expression of E3α-II is induced by IL-6 and TNF-α, thus suggesting another link between systemic inflammation and muscle wasting [362].

The E3 ligases may also play roles in the development of cancer, by functioning as either oncogenes or oncosuppressor genes. Examples of oncogenic ubiquitin ligases include HectH9, which regulates transcriptional activation by Myc [363] and is essential for tumour cell proliferation, and MDM2, which regulates p53 inactivation [364]. An example of oncosuppressor activity is that of the *vHL (von Hippel-Lindau)* gene on the short arm of chromosome 3 [365]. The main function of the encoded protein is an E3 ubiquitin ligase which targets (amongst others) hypoxia inducible factor (HIF) 1a, a transcription factor that controls the expression of a number of angiogenic proteins. Loss of VHL protein activity results in an increased amount of HIF1a, increased levels of angiogenic factors, and the development of

VHL syndrome, a condition characterised by multiple benign and malignant vascular tumours [365].

Increased activity and expression of the UPP has been demonstrated in several human disease states associated with muscle wasting, including sepsis with multiple organ failure, and cancer [164, 225, 366]. Specifically, ubiquitin mRNA levels and proteasome proteolytic activities (chymotrypsin-like, trypsin-like, and peptidyl-glutamyl-peptidase) were increased in muscle samples from gastric cancer patients [367, 368], whereas levels of ubiquitin mRNA, proteasome subunit (C2 and C5) mRNA and ubiquitin conjugating enzyme E2(14k) protein were correlated with weight loss in pancreatic cancer patients [225, 360].

When considering the efficacy of an upstream approach to preventing muscle catabolism, it should be remembered that the consequences of chronic impairment of the systemic inflammatory (and immune response) in patients with underlying cancer are currently unknown. Trials of IL-1 receptor antagonists (e.g. anakinra) and TNF inhibitors (e.g. infliximab, etanercept) in rheumatoid arthritis have been complicated by increased rates of opportunistic infections (particularly reactivation of tuberculosis in the case of infliximab) [369]. Therefore, a more selective downstream approach would be safer and more effective at attenuating muscle loss. In this respect, direct proteasome inhibition could prove an effective strategy. Experimental pharmacological agents that could achieve this aim include peptide aldehydes, lactacystin/β-lactone, vinyl sulphones and dipeptide boronic acid analogues [370]. Bortezomib is an example of the latter type of agent and a

selective inhibitor of the 20S proteasome [370]. Marketed as *Velcade*, it is now a recognised anti-tumour treatment for patients with relapsed, treatment-refractory multiple myeloma [371]. The main modes of action of bortezomib in inducing tumour cell death appear to be the regulation of NF-κB activity [372], the induction of apoptotic pathways [373], and the induction of autophagy [374]. Unfortunately, the current evidence for proteasome inhibitors as a means of reducing human muscle wasting is less encouraging. Despite rat studies demonstrating a significant reduction in denervation-induced muscle atrophy following bortezomib administration [375], preliminary studies in human patients with metastatic pancreatic cancer have demonstrated an insignificant impact on weight loss [376]. Moreover, despite being a selective downstream approach, bortezomib may still be too non-specific to be a successful intervention in cancer cachexia. Global inhibition of proteasome activity will impact on a wide range of regulatory, proteolytic systems (other than just muscle atrophy), and therefore the side effect profile is likely to be prohibitively toxic. At doses used for the treatment of multiple myeloma, asthenia and anorexia, two undesirable symptoms of cachexia, are two of the commonest side effects associated with bortezomib administration [371]. Thus, a targeted approach to UPP inhibition (e.g. enzyme inhibitor of MAFbx or MURF-1) would be superior.

1.9.2.5 Tripeptidyl peptidase

Tripeptidyl peptidase (TPP) is a high molecular weight peptidase that sequentially removes tripeptides from free N-termini of oligopeptides generated by the proteasome. It may also have a low-grade endopeptidase action and play a role in

the degradation of specific cellular substrates. TPP activity was upregulated in sepsis-induced muscle wasting [377]. In the MAC16 mouse tumour model, both proteasome activity and TPPII activity increased with increasing weight loss, reaching a maximum at 16% weight loss, after which there was a progressive decrease in activity of both proteases [378].

In summary, over the previous six sections, it has been shown that numerous processes may contribute to weight loss in cancer. With regard to the key target of skeletal muscle, inflammatory, neuroendocrine and tumour-derived mediators appear to act through a variety of intracellular proteolytic processes that have been summarised in this chapter. In particular, the IGF-1/PI3K/Akt pathway has been identified as a key anabolic signalling mechanism, whereas the NF-κB and FOXO transcription factors have been acknowledged as crucial mediators in the transcriptional regulation of atrogenes. The need for confirmation of whether or not DGC deregulation is involved in human cancer cachexia has been recognised. When considering intracellular effector mechanisms of muscle wasting, the UPP has been identified as a key apparatus of protein degradation, and the emerging roles of caspases and autophagy/lysosomal machinery have been described (Figure 1.15). At present, no studies have been performed in cachectic cancer patients to assess the final outcome of degraded myofibrillar proteins. However, this thesis explores the fate of degraded proteins that enter the systemic circulation and are excreted in urine, in order to assess their usefulness as biomarkers of skeletal muscle wasting (Chapter 9). This section represents the last to detail the aetiology

of cancer cachexia. The following section considers the clinical consequences of cancer cachexia.

1.10 Clinical consequences of cancer cachexia

The objective clinical consequences of cachexia are profound including shortened survival [4], impaired response to anti-cancer therapy [4], impaired immunity [379], reduced performance status (PS) [380], reduced PA [380] and worsened QoL (Figure 1.16). Specific examples of studies that support these assertions are detailed below. There is a *prima facie* case that depletion of skeletal muscle is the key component of weight loss that underpins many of the symptoms experienced by cachectic cancer patients. Muscle wasting leads to reduced exercise capacity and increased fatigue [381], which results in the lower PS scores, reduced PA and worsened QoL scores observed. Furthermore, there does not appear to be an alternative explanation by which loss of fat mass could account for such findings. Thus, skeletal muscle wasting is the critical driver behind patient symptomatology in cancer cachexia. This supposition is supported by studies that have demonstrated that sarcopenic obesity (i.e. skeletal muscle wasting in the ongoing maintenance of fat mass) in cancer patients was associated with poorer functional status compared with obese patients who did not have sarcopenia [382].

The mechanism by which skeletal muscle wasting is linked to the actual mode of death of the cachectic patient is not proven. However, the most common cause of death in cancer patients is infection and respiratory failure [383], presumably as a result of wasting of the respiratory muscles. Furthermore, patients with nutritional

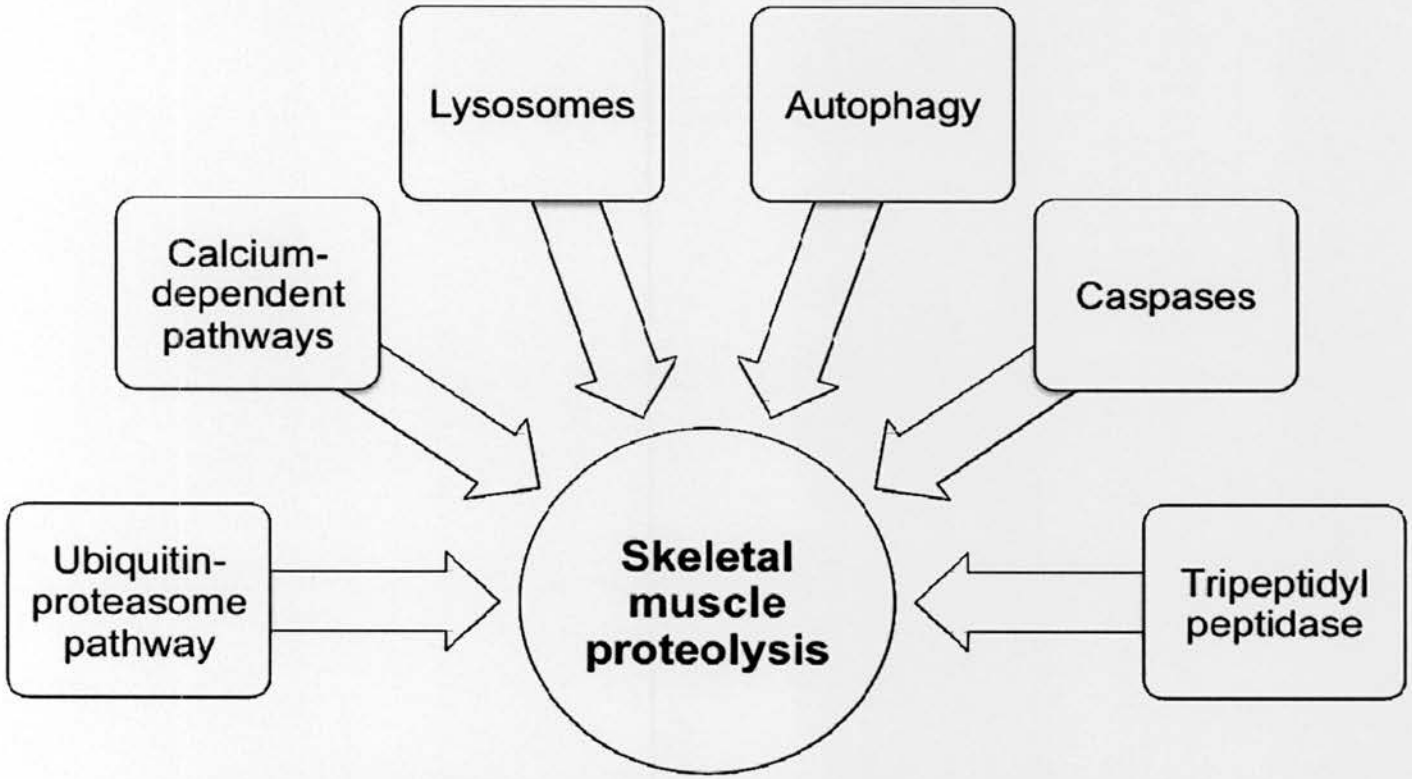

Figure 1.15 **Intracellular effector mechanisms of proteolysis in skeletal muscle.**
Numerous intracellular mechanisms of proteolysis exist within skeletal muscle. The UPP represents a key apparatus of protein degradation. Calcium-dependent pathways and caspases may act upstream of the UPP, whereas TPP may act downstream. The importance of autophagy and lysosomal machinery within human muscle wasting is not fully understood currently but may represent another crucial degradative mechanism.

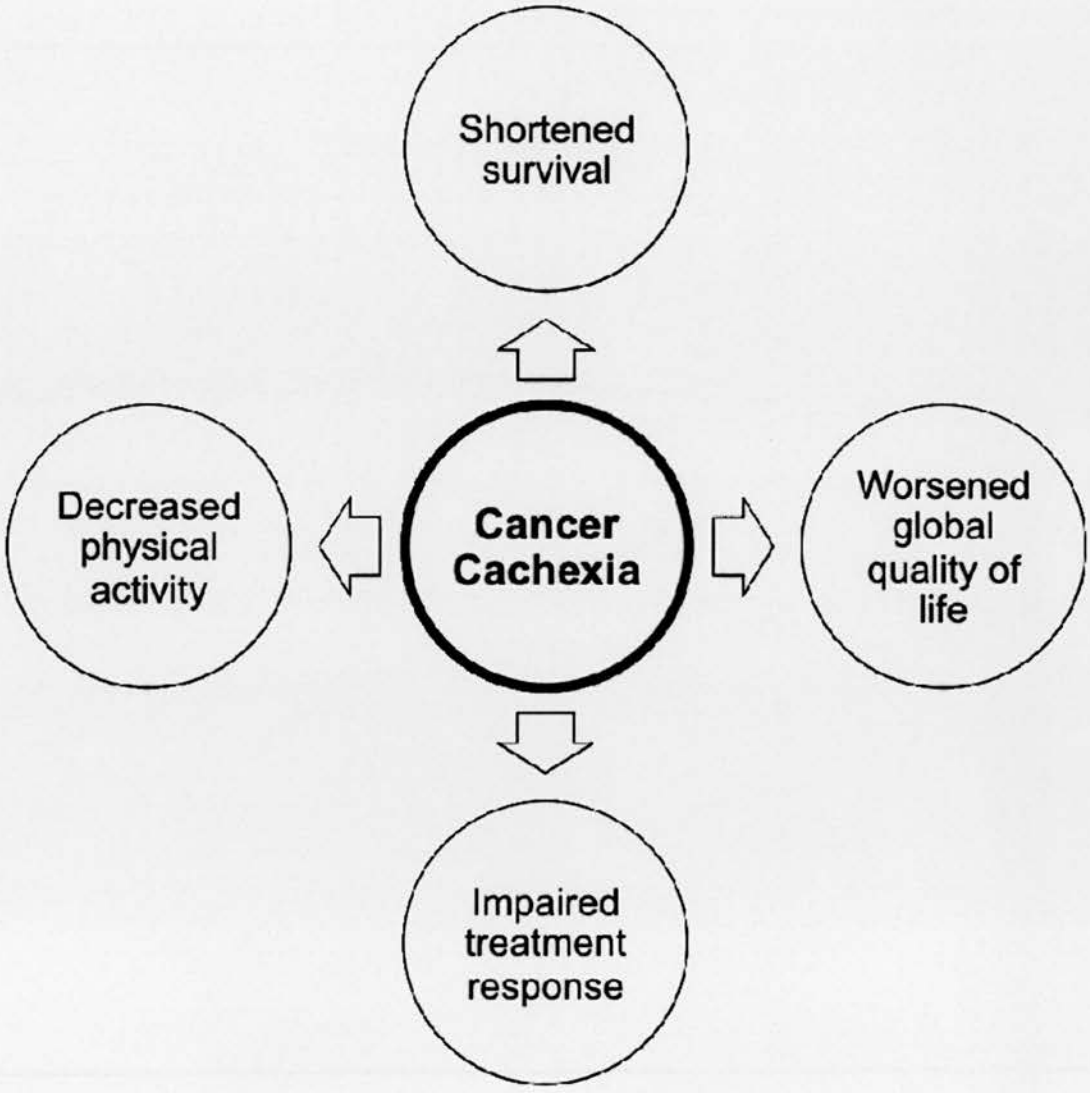

Figure 1.16 **Clinical consequences of cancer cachexia.**
Reduced survival, impaired response to anti-cancer therapy, impaired immunity, lower performance status scores, increased symptomatology, reduced PA and worsened QoL scores have all been demonstrated in association with cancer cachexia. *Prima facie* evidence would state that skeletal muscle wasting is an important determinant of outcome within cachexia that results in the reduced PS scores, decreased PA, worsened QoL scores, and, presumably, shortened survival experienced by cachectic cancer patients.

depletion and less physiological reserve are known to be more susceptible to complications [4], which will deplete nutritional status further, and ultimately may have fatal consequences (Figure 1.17).

1.10.1 Effect on functional status and quality of life

The functional consequences of cancer cachexia have been discussed briefly in Chapter 1.8.2, in which the level of PA of patients with cancer cachexia was seen to be as low as that observed in patients with spinal cord injury [280]. In addition, there exist many other studies in the literature that demonstrate the impact of cachexia on PF and QoL.

In a study of patients with inoperable NSCLC, approximately 40% had lost at least 5% of pre-morbid weight, and almost 80% exhibited an elevated CRP [384]. Weight-losing patients suffered greater fatigue and pain and demonstrated significantly lower KPS and overall QoL scores than weight-stable counterparts [384]. An elevated CRP was also associated independently with increased fatigue.

In another 1-month study of 24 patients with lung cancer, KPS and self-reported tools were used to show that PF deteriorated in those not receiving chemotherapy [385]. Thirty three percent of patients experienced difficulty walking one block or more, 79% complained of serious fatigue and 44% had difficulty with household chores [385]. Only 21% of patients were completely satisfied with their level of activity.

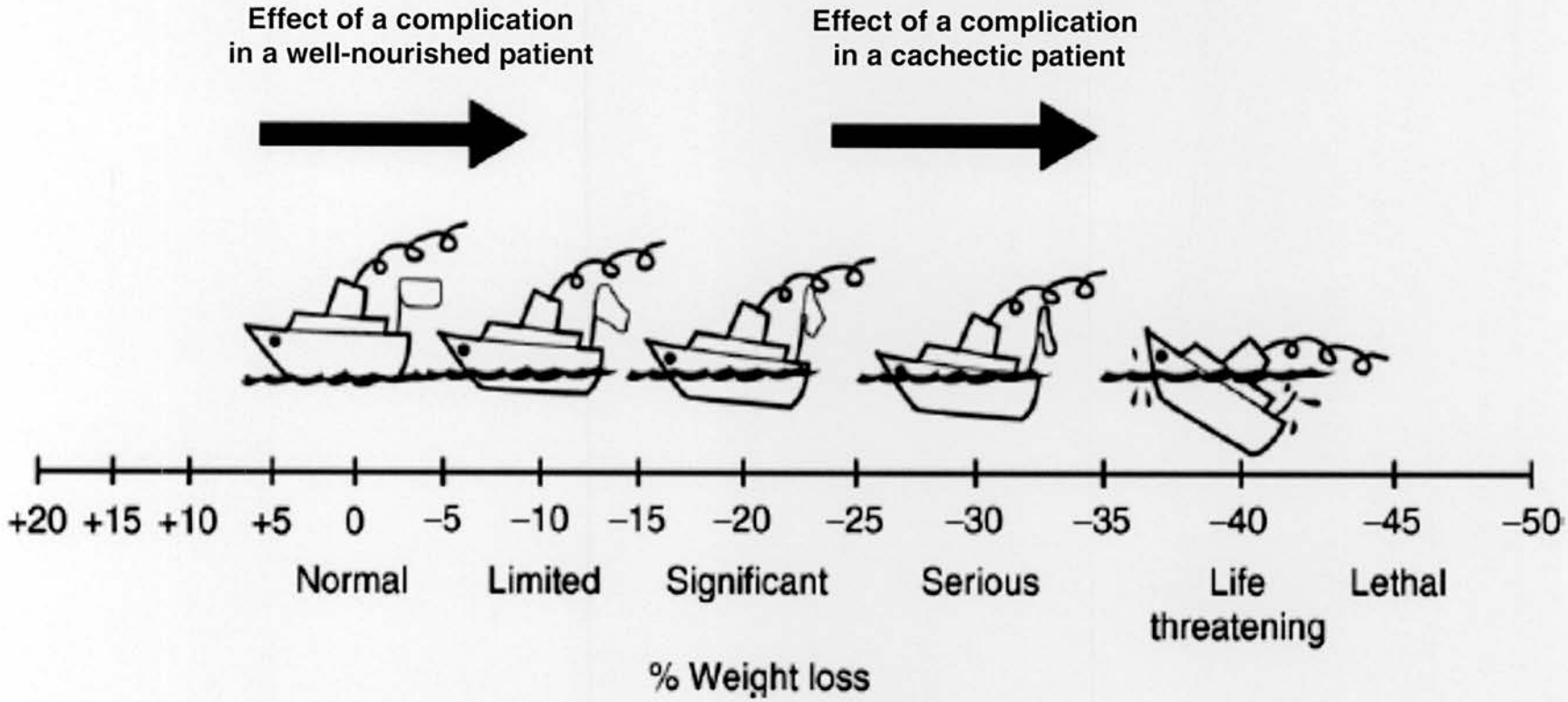

Figure 1.17 **Effect of nutritional depletion on patient outcome.**
As weight loss increases, the risk of adverse outcome for the patient also increases, until eventually, death ensues. Cachectic patients are also at increased risk of complications, which will deplete nutritional status further, and push patients further along the path to death.

In another population of 100 ambulant patients with advanced NSCLC, PF was assessed both subjectively (KPS) and objectively (Simmonds Functional Assessment tool [SFA] and six-minute walk) [386]. The authors concluded that measurable differences in daily tasks were identifiable when compared with a normative population. There was only low to moderate correlation between these abnormalities and subjective fatigue tests, suggesting that objective measures of PF provide important and independent endpoints for clinical trials and therapeutic interventions in this population [386]. This notion is discussed in detail in Chapter 1.12.

In a series of studies on patients with advanced GI cancer, patients with more than 5% weight loss had a higher CRP, lower albumin, lower anthropometry measures, poorer appetite and reduced QoL scores [387-389]. The authors calculated that a loss or gain of >2.5kg over 6-8 weeks was required to produce a significant change in PS in weight-losing patients [388]. Both reduced PS and elevated CRP were also associated independently with survival [389].

In patients with advanced GI cancer receiving palliative chemotherapy, weight loss was associated with reduced global QoL [390]. In addition, weight loss and poor PS were related independently to lower probability of treatment response and decreased survival on multivariate analysis [390].

1.10.2 Effect on response to palliative therapy

A retrospective review of 1555 patients receiving chemotherapy for GI malignancy determined that patients with weight loss received lower initial drug doses but

developed more frequent and severe dose-limiting toxicity: on average, they received 1 month less treatment [391]. Furthermore, weight loss correlated with reduced failure-free and overall survival. Patients who stopped losing weight had an improved overall survival. The authors concluded that these results provided a rationale for randomised nutritional intervention studies in this population [391]. Comparable results have been found in studies of lung cancer. NSCLC or mesothelioma patients with weight loss failed more frequently to complete at least 3 cycles of chemotherapy, and experienced anaemia more frequently as a drug-associated toxicity [33]. In similar patients, the Voorrips Physical Activity Questionnaire (PAQ) was also shown to be predictive of World Health Organisation (WHO) grade 3 or higher toxicity, but not 90-day disease progression [392].

In women with metastatic breast cancer resistant to anthracycline and/or taxane treatment, CT body composition analysis demonstrated that capecitabine-associated toxicity was present in 50% of sarcopenic patients, compared with only 20% of non-sarcopenic patients [393]. Furthermore, time to tumour progression was shorter in the sarcopenic patients [393].

1.10.3 Effect on survival

Weight loss and KPS have long been known to be predictive of survival. In the ECOG's paper of 1980 [4], weight loss prior to chemotherapy was noted be a significant prognostic factor in a wide range of malignancies. Within each of the 12 cancer types investigated, survival was shorter in the patients who had experienced weight loss compared with patients who had not. For sarcoma, unfavourable non-

Hodgkin's lymphoma, colon and prostate cancer, the median survival was approximately twice as long in the weight-stable patients compared with weight-losing patients [4]. When the data were analysed by weight loss categories, the greatest difference in survival was noted between the 0% weight loss and the 0-5 % weight loss categories, suggesting that the *presence* of cachexia is more important as a marker of survival than the *severity* of the cachexia at presentation. Weight loss also correlated with deterioration in PS in 11 of 12 cancer types [4].

The importance of the APPR as a prognostic variable in cancer patients has been discussed previously in Chapter 1.4.2.1. However, the exact magnitude of the impact on survival was not described, and potentially this effect can be very clinically significant. For example, in patients with gastric cancer, those with an APPR exhibited a severely shortened median survival duration compared with patients without evidence of systemic inflammation (9 weeks versus 53 weeks) [394].

In this section, the clinical consequences of cancer cachexia, including decreased QoL scores, poorer functional status and impaired physical activity, and shortened survival, have all been summarised. In the following section, general approaches for the management of the cachectic cancer patient are outlined, including other potential nutritional and therapeutic interventions not yet discussed.

1.11 Management of cancer cachexia

The management of cancer cachexia requires a multimodal approach involving a dedicated multidisciplinary team consisting of physician/oncologist, surgeon, general practitioner, nurse specialist and dietician. This team should follow patients regularly as cachexia is a progressive condition that requires repeated evaluation over time (Figure 1.3). At present, there is no single therapeutic intervention of proven benefit in all cachectic cancer patients. Instead, in the future, it is likely that a combination of different treatments will be required simultaneously to reverse wasting successfully (Figure 1.18).

1.11.1 Symptom control and supportive care

Cancer and cachexia can be associated with a wide range of distressing, yet reversible, symptoms. Efforts should be taken to ameliorate these symptoms wherever possible, in order to maximise patient QoL and create an ideal background for the optimisation of appetite and the reversal of the underlying metabolic disorder. Nausea and vomiting should be controlled with regular anti-emetics (or surgery for mechanical obstruction); early satiety can be improved with gastric prokinetic agents (e.g. metoclopramide); malabsorption is treated with pancreatic enzyme supplements; and constipation is relieved by laxatives. Whenever pain is a significant issue, attempts should be made to control it with the minimum of sedation. The diagnosis of major depression in cachectic cancer patients is often clouded by neurovegetative symptoms that may be secondary to either cancer or depression. However, in those cases where depression is predominant, anti-depressant medication and/or counselling reduce dysphoria [395].

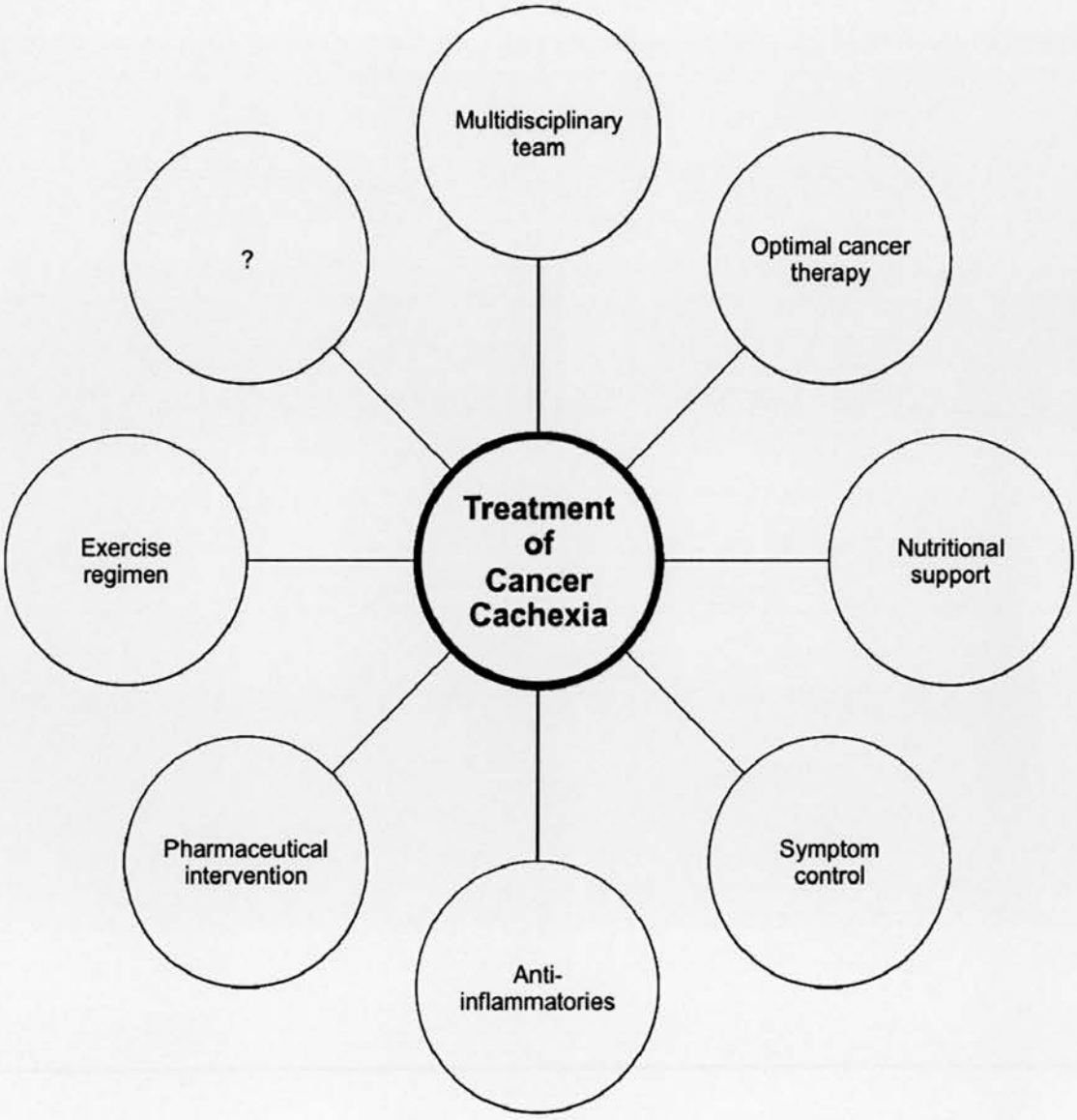

Figure 1.18 **Combination therapy for patients with cancer cachexia.**
A constellation of different pharmaceutical, nutritional, psychological and exercise-related treatments, all under the regulation of a dedicated multidisciplinary team, are required if cachexia is to be managed successfully. However, as there is presently no one accepted or universally effective management strategy, questions still remain.

1.11.2 Nutritional therapies

It is now more than 25 years since Nixon and co-workers observed a suboptimal response to nutritional support in cancer patients [2, 396]. In this study, weight-losing cancer patients who were treated with enteral nutritional support demonstrated significantly less improvement in body weight, serum albumin level, creatinine/height ratio, and mid-arm muscle area, compared with weight-losing non-cancer controls [2]. Thus, there appeared to be a partial anabolic blockade to the accretion of lean tissue in advanced cancer patients. In contrast, gain in fat mass was the same in the wasted cancer and non-cancer patients [2]. These findings demonstrate that although the negative energy balance in cancer cachexia can be overcome by artificial nutritional support, it appears to be much more difficult to prevent or reverse the loss of LBM. However, despite the fact that nutritional support cannot represent the whole solution to cancer cachexia, ensuring that the cachectic patient receives adequate nutritional intake is of paramount importance. One interesting study examined the features of cachexia that correlated with survival following a 4-month follow-up of cancer patients undergoing outpatient palliative care (n=297) [397]. In this patient group, weight loss and persistent hypermetabolism were found to be associated significantly with a shorter duration of survival. The baseline nutritional intake of the patients at the start of the study was 26kcal/kg/day or approximately 1560kcal/day [397]. Dietary intake did not differ between normo- and hypermetabolic patients, nor was tumour type or gender related to energy and protein intake. Patients able to increase their energy intake demonstrated a significant prolongation of survival [397]. This study therefore identified a daily food intake >1500kcal as a favourable prognostic variable. The underlying reason for this observation is apparent from recent investigations of the

individual components of TEE. In patients with advanced pancreatic cancer and weight loss, average TEE was 1700kcal/day, average REE was 1400kcal/day, and average food intake was 1500kcal/day [277]. Thus, the net result was a persistent negative energy balance of approximately 200kcal/day per patient. In this scenario, it seems that any increase in PA towards normal levels, in the absence of nutritional supplementation, would simply worsen the established negative energy balance. In contrast, any increase in food intake from approximately 1500kcal/day towards at least 1700kcal/day would result in the restoration of positive energy balance, which might prolong the patient's ultimate survival.

The Society for Sarcopenia, Cachexia, and Wasting Disease recently convened an expert panel to develop nutritional recommendations for the prevention and management of skeletal muscle wasting [398]. They identified that exercise (both resistance and aerobic) in combination with adequate protein and energy intake were the key components to any successful strategy. In particular, it was stressed that adequate protein intake (including leucine-enriched balanced amino acids and possibly creatine) may enhance muscle strength [398]. They also recommended vitamin D replacement where measured levels were low. However, in practice, improvement of nutritional intake within the cachectic patient is difficult to achieve. Early and formal nutritional advice should be sought from a dietician, and if necessary, intensive nutritional counselling. Nutritional intake can be improved by providing small, frequent meals that are energy-dense and easy to consume (e.g. dairy products). The patient should avoid extremes of taste and smell, and meals with high fat content (fat delays gastric emptying). Moderate alcohol consumption

before and during a meal can help. Provision of energy and protein-dense oral supplements can be beneficial if taken regularly, but patient compliance is often a problem. Patients should aim to take 200-400mls of supplements daily (300-600kcal) accepting that this may suppress some normal food intake but provide an overall gain of 200-400kcal/d. In some advanced cancer patients, the decision may be taken to pursue artificial nutritional support (either enteral or parenteral). Recent studies of an integrated artificial nutrition approach to cancer patient care, involving COX inhibitor treatment, rhEPO and either oral nutritional support or home total TPN, have had success at improving patient energy balance and higher maximum exercise capacity [399]. However, during such an approach, it is important to consider the ethical balance between benefit in QoL and the potential problems associated with artificial nutritional support (e.g. time spent in hospital, potential complications of central venous access for TPN).

The above two paragraphs describe general recommendations for nutritional support in the cachectic patients. However, two nutritional supplements have received particular attention in the treatment of cancer cachexia and these are discussed below.

1.11.2.1 Eicosapentaenoic acid

EPA is an omega-3 polyunsaturated fatty acid (PUFA), the main role of which is exercised during inflammation [400]. Membrane-bound EPA and AA (i.e. 20 carbon fatty acids) are used as precursors by COX and lipoxygenase (LOX) to generate physiologically active eicosanoids. COX activity produces prostaglandins

and thromboxanes whereas LOX activity produces leukotrienes [400]. The majority of eicosanoids are generated from AA, as it exists in much greater amounts than EPA within the phospholipid cell bilayer. AA-derived eicosanoids (2-series prostaglandins) are also more potent inducers of inflammation than their EPA-derived counterparts (3-series prostaglandins). Thus, EPA-derived prostaglandins are less favourable for the development of cachexia. EPA is also known to have a number of specific anti-cachexia effects, including impacts on tumour differentiation, lipid metabolism, tumour-derived mediator function, pro-inflammatory gene transcription and cytokine expression [400]. Furthermore, in pre-clinical models, it has demonstrated synergistic anti-cancer effects with conventional chemotherapeutic treatments. In the MAC16 murine cachexia model, EPA treatment resulted in an 88% decrease in muscle protein degradation (but had no effect on protein synthesis) [401]. The presumed mechanism behind these observations is EPA-induced inhibition of the UPP, possibly via prevention of formation of 15-hydroxyeicosatetraenoic acid (15-HETE). In rats bearing the methylcholanthrene (MCA) fibrosarcoma, it has been shown that EPA supplementation can decrease UPP components in both skeletal muscle and liver [402]. Specifically, skeletal muscle demonstrated decreased E2(14k) and E3α expression, whereas hepatic tissue demonstrated decreased C2 mRNA expression [402]. The observed differences in the hepatic and muscle gene expressions of UPP components suggested an organ-specific effect for omega-3 fatty acid activity.

Initial clinical studies of EPA in cachectic cancer patients were encouraging. When administered either as fish oil capsules (12g EPA/day) or as a purified fatty acid (6g EPA/day), EPA was shown to arrest the development of cachexia in weight-losing

patients with pancreatic cancer [403]. Furthermore, a multimodal approach combining oral nutritional supplementation and EPA (2.2g/day) in patients with pancreatic cancer resulted in net gain of weight (median weight gain of 1 kg at 3 weeks) and LBM, along with an improvement in appetite and performance status [404]. The same combination was also shown to increase patients' PAL (although this effect did not require a significant increase in LBM) [277] (see Chapter 1.8.2, p.92) and decrease the stimulatory effect of feeding on the hepatic APPR (see Chapter 1.8.3, p.95) [285]. A further study of 27 pancreatic cancer patients undertaking a daily administration of 6g of EPA observed a reversal of weight loss associated with decreases in serum levels of CRP, IL-6 and TNF-α [405]. However, despite these early positive results, larger randomised clinical trials have been less successful. One such trial in cachectic pancreatic cancer patients (n=200) was compromised by issues of non-compliance (although post-hoc dose-response analysis did demonstrate a linear relationship between plasma EPA and gain in LBM) [406], whereas in another trial of patients with advanced GI or lung cancer (n=518), both intention-to-treat analysis and per protocol analysis revealed no significant improvements in survival or weight [407]. In a further study of patients with non-hormone-dependent types of cancer (n=421) treated with a combination of MA and EPA, no significant improvement in weight gain, appetite or QoL was observed over MA alone [408]. Ultimately, a recent Cochrane Database System Review has concluded that there are currently insufficient data to establish whether or not oral EPA is superior to placebo [409]. However, once again, the failure of efficacy of EPA may be due to a lack of patient targeting, a poor understanding of dose-response relationships, and a need for combination strategies. For example, certain combination strategies using EPA have been more efficacious. In one study

of solid tumour patients (n=60) assigned randomly to receive a daily fish oil supplement that contained EPA (18g of omega-3 PUFAs) with vitamin E (to compensate for the oxidative effect of the fish oil), there was a doubling of median survival in the EPA-supplemented arm compared with placebo [410]. Furthermore, in another trial of patients with advanced lung cancer (n=22), patients receiving fish oil in combination with celecoxib (a COX-2 inhibitor) demonstrated lower CRP levels, greater muscle strength and body weight compared with patients receiving fish oil and placebo [411].

1.11.2.2 β-hydroxy-β-methylbutyrate

Another nutritional agent that has been investigated in patients with cancer cachexia is β-hydroxy-β-methylbutyrate (HMB), a derivative of the branched-chain amino acid (BCAA) leucine. It has long been known that TPN supplemented with BCAA causes improved protein utilisation and synthesis in cancer patients [412]. In experimental murine models, HMB was capable of increasing muscle protein synthesis via increased phosphorylation of mTOR, $p70^{s6k}$ and eIF4E-BP1 [413], reduced expression of UPP components [414], and reduced proteolysis [414]. Early clinical trials of HMB administered with arginine and glutamine increased FFM, improved emotional profile, and decreased subjective feelings of weakness in wasted cancer patients [415, 416]. However, the largest trial to date of weight-losing cancer patients (n=472) was unable to prove conclusively an increase in LBM, possibly due to the fact that the majority of patients dropped out of the study prior to completion, leaving only 37% of participants to finish the 8-week protocol [417].

1.11.3 Exercise regimens and modulators of fatigue

Fatigued patients with significantly reduced energy reserves should be advised to make efficient use of the energy they do have by focusing on meal times and social interaction. Increasing psychosocial support and limiting patient stress levels may further reduce fatigue, whereas input from occupational therapists and the provision of physical aids in the home may also improve mobility. Regarding therapeutic interventions aimed at improving exercise capacity in advanced cancer patients, prescribed regimens of physical exercise and/or drugs that increase haemoglobin concentrations or modulate fatigue centrally have all been trialled.

1.11.3.1 Exercise regimens

Exercise regimens have been shown to improve QoL, PF, LBM, mood, emotional well-being and social well-being in palliative cancer patients [418]. Moreover, in the absence of such programmes, advanced cancer patients have expressed interest in exercise regimens and have stated that they would feel able to participate in them [419]. In particular, such patients have exhibited strong preferences for home-based PA programmes that could be completed alone, including neuromuscular electrical stimulation (NMES) [420], walking and resistance training being the most popular types of activity [419]. However, despite such stated interest, an invitation to undertake a regimen of therapeutic exercise is only accepted by approximately two-thirds of cancer patients, and only completed by approximately one half [418].

At present, a number of different exercise regimens have been trialled, including resistance training [421], aerobic training [422], yoga [423] and NMES [418]. Yet the superior type of exercise, the form it should take, the intensity, and the timing of

said exercise remain unclear. Furthermore, despite patient enthusiasm, the feasibility of commencing successful exercise regimens, particularly resistance training, in elderly, frail patients is uncertain.

1.11.3.2 Fatigue modulators

If fatigue occurs in the presence of anaemia, rhEPO treatment may be effective at restoring haemoglobin concentration and improving exercise capacity, although the benefit derived may not solely arise from its haematopoietic effects. There is increasing evidence for a protective role of EPO in experimental models of both myocardial infarction and ischaemia/reperfusion injury through anti-apoptotic effects within the myocardium [424, 425]. However, there remain concerns regarding the effect of chronic EPO treatment on the progression of neoplastic disease [426]. In studies of head and neck cancer patients with anaemia, long-term rhEPO therapy significantly impaired loco-regional disease control and shortened survival [427]. For this reason, a Cochrane Database System review on the management of cancer-related fatigue does not recommend the use of rhEPO in these patients [428]. However, the authors did suggest that psychostimulants, such as methylphenidate, represent alternative pharmaceuticals that may be used to decrease fatigue, which avoid the potential dangers associated with EPO [428].

1.11.4 Pharmaceutical intervention

In the previous sections dealing with the aetiology of cancer cachexia, a number of therapeutic interventions that have been or are being trialled for cancer cachexia have been described, including cytokine antagonists, SARMs, ACE inhibitors, β-agonists, myostatin antagonists, GH, ghrelin analogues, and proteasome inhibitors.

In the present section, pharmaceutical interventions that have been trialled in cancer patients but not described previously in this thesis are discussed. Such therapies interrupt the wasting process by either reducing anorexia/stimulating appetite or by attenuating skeletal muscle catabolism.

1.11.4.1 Progestational agents and hormonal appetite stimulants

In patients complaining of severe anorexia or early satiety, an appetite stimulant may provide symptomatic improvement. Progestational agents (e.g. MA or medroxyprogesterone) at high doses can improve appetite in approximately 70% of patients [429, 430] by downregulating pro-inflammatory cytokines [431]. However, despite subjective improvements in appetite, increased food intake and weight gain may only be observed in approximately 20% of patients [432-434]. The dissociation between subjective appetite scores and objective improvement in nutritional status may be the product of three particular problems associated with progestational agents. Firstly, any observed weight gain is often due to oedema or increased fat deposition, rather than skeletal muscle [435, 436]. Furthermore, by reducing circulating androgen levels, progestagens might actually decrease skeletal muscle mass [437]. Secondly, the exact dose of progestagens required is unknown, and trial doses have ranged from 160 to 1600mg/day [438]. Thirdly, there are a significant number of potential side effects that arise from these treatments, including thromboembolism [439], hyperglycaemia, hypertension, peripheral oedema, alopecia and adrenal insufficiency. A recent Cochrane review concluded that MA can increase appetite and weight gain in cancer patients, but that there is not yet sufficient evidence to reach a conclusion about the optimal dose or the effect on QoL [440]. Other hormonal appetite stimulants available include

corticosteroids (e.g. prednisolone, dexamethasone) and ghrelin (see Chapter 1.7.1.4, p.87). Corticosteroids can induce a transient effect on appetite, PS and subjective physical well-being, but these sensations are limited usually to a few weeks duration [441, 442].

1.11.4.2 Cannabinoids

Cannabinoids represent an alternative group of pharmaceutical agents known to increase appetite through their interaction with CB_1 cannabinoid receptors within the hypothalamic ARC [443]. However, two published trials regarding the use of cannabinoids in cancer-associated anorexia were largely unsuccessful. The first study, which compared the use of cannabis extract, δ-9-tetrahydrocannabinol (THC), and placebo on appetite and QOL in advanced cancer patients (n=243), was terminated because of insufficient differences between study arms [444]. The second study, which compared dronabinol alone, MA alone, and a combination therapy of the two drugs on appetite, nutritional status and QoL in cachectic advanced cancer patients (n=469), concluded that MA provided superior palliation of anorexia compared with dronabinol alone, and that combination therapy conferred no additional benefit [445].

1.11.4.3 Non-steroidal anti-inflammatory drugs and COX-2 inhibitors

Early attempts at COX inhibition in cachectic cancer patients using non-steroidal anti-inflammatory drugs (NSAIDS) were encouraging. In one study, indomethacin (50mg twice daily) was capable of doubling mean survival duration in malnourished patients with solid tumours (n=135) [446]. Furthermore, subsequent studies demonstrated reductions in systemic inflammation and REE [447, 448].

However, a positive impact on survival has not yet been replicated, possibly due to the fact that NSAIDs appear able to add or maintain fat mass but not LBM [447].

Recently, researchers have favoured selective COX-2 inhibitors [449], rather than NSAIDs, as they avoid the unwanted risk of peptic ulceration. (Fears still surround the potential for cardiac-associated toxicities following chronic COX-2 inhibition [450], but these latter side effects are likely to be less significant in palliative cancer patients with short life expectancies). For reasons that are not yet clear, these second-generation medications may be capable of improving LBM, whereas NSAIDs were not. A 4-month treatment course of celecoxib (300 mg/day) in patients with advanced cancer was well tolerated and resulted in significant increases of LBM, grip strength, QoL, and PS [449]. COX-2 inhibitors are already being employed within multimodal anti-cachexia treatment stategies [451].

1.11.4.4 Thalidomide

Thalidomide is well known for its teratogenic effects but it is licensed currently for the treatment of multiple myeloma [452]. The treatment effects of thalidomide are not explained fully, but appear to be the result of its immunomodulatory and anti-angiogenic properties [453]. The proposed anti-cachectic mechanism of thalidomide is an inhibition of TNF-α synthesis [454], although reduction in serum IL-6 and CRP levels have also been demonstrated [455]. In a cachectic rat cholangiocarcinoma model, thalidomide treatment preserved fast-twitch skeletal muscle fibres and was associated with decreased expression of TNF-α and TGF-β [456].

Both thalidomide and the thalidomide analogue, lenalidomide [457], have been trialled for use in patients with advanced solid tumours. In advanced pancreatic cancer patients (n=50), thalidomide was able to prevent loss of arm muscle mass and patient weight after both 4 and 8 weeks of therapy [458]. In a further study of patients with non-obstructing but inoperable oesophageal cancer (n=11), a 2-week treatment course of thalidomide (200mg daily) was capable of reversing the loss of weight and LBM experienced by patients established on an isocaloric diet for 2 weeks previously [459].

1.11.4.5 Adenosine-5'-triphosphate

Extracellular ATP is involved in the regulation of a variety of energetic biological processes, including muscle contraction, neurotransmission and liver glucose metabolism via purinergic receptors [460]. It has therefore been hypothesised that ATP infusion may reverse the negative energy balance found within cachectic cancer patients [461]. In clinical trials, ATP infusion inhibited the depletion of body composition and the loss of QoL in patients with advanced NSCLC [462], but did not alter REE [461]. Interestingly, ATP infusion appeared to be especially beneficial to those patients who were identified as cachectic (defined as weight loss $\geq$5%), perhaps because the energy imbalance in these patients is at its most profound [462, 463]. In these individuals, ATP infusion was shown to be most effective at restoring liver energy balance [463]. Furthermore, ATP was able to prolong the survival of weight-losing patients with stage IIIb NSCLC, but not weight-stable patients or patients with stage IV NSLC [464, 465].

In the previous section, the multimodal approach to any cancer cachexia therapeutic intervention has been stressed. Furthermore, examples of clinical trials that have investigated anti-cachexia interventions have been cited. Many of these trials have been largely unsuccessful, probably because most of the interventions have been unimodal in nature. However, several other problems that confound the current design of anti-cachexia clinical trials will also have contributed to the generation of negative findings, not least the difficulties in defining and diagnosing cachexia and the lack of agreed biomarkers (see Chapter 1.2, p.34). In the next section, the complexities of cancer cachexia trial design are discussed, and, in particular, the use of outcome measures.

1.12 Outcome measures in cancer cachexia

The challenge in clinical trials of cancer cachexia is to develop an intervention of sufficient efficacy that, not only does the measured nutritional status of patients improve significantly, but that the effect of this improved nutritional status on adverse clinical outcomes can also be demonstrated. Cachexia becomes even more complex when we attempt to judge the success or failure of any proposed therapy for its amelioration. The United States Food and Drug Administration (FDA) has decreed that, in order for a treatment to be licensed for the management of cachexia, there must be a documented improvement in both the nutritional and functional status of a patient so treated [466]. To achieve this aim, any primary outcome measure used in clinical trials should ideally be a biomarker of both nutritional and functional status. This section details a critical evaluation of the use of outcome

measures in cancer cachexia. In particular, the objective assessment of PA is proposed as a clinical biomarker of nutritional status, functional status and QoL.

1.12.1 Reaching a consensus on primary outcome measures

Outcome measures are "*the results of health care processes*" [467]. They are a measure of change and represent the difference from one point in time (usually before an intervention) to another point in time (usually following an intervention) [468]. Outcome measures should be standardised, with explicit instructions for administration and scoring [469].

When developing a *primary outcome measure* for studies in cachexia, it is crucial to remember that it should represent the outcome of prime importance. As such, it should be a significant and relevant end-point, which is clearly stated and defined. It should also have a reasonable chance of being proven (c.f. power analysis) by the proposed intervention trial. In contrast, a *surrogate outcome measure* is an observational marker that is believed to relate to the primary end-point. It can be clinical, physiological, chemical or biological. Surrogate outcomes should be easy to identify and should be a good proxy or true predictor of the primary outcome.

The primary clinical outcome measures that have been used in clinical nutrition trials include variables that are either patient-, doctor- or service-focused. Patient-focused outcomes may include QoL variables [470] or measures of patient independence [430]. Doctor-focused outcomes include measures of patient survival [432], the incidence of complications [438] and the length of hospital stay [471]. Service-focused outcomes include assessments of the cost effectiveness of any new

treatment and the financial viability of the introduction of such a treatment to medical practice [472]. Unfortunately, the direct measurement of any of these variables is often a difficult and costly exercise. This problem, compounded by the multiple potential confounding influences on such outcomes, renders a trial of appropriate statistical power dauntingly large, complex and expensive. This has often led investigators and pharmaceutical companies to abandon the direct measurement of adverse clinical outcomes in favour of simpler, cheaper studies. For example, if assessing the efficacy of a new nutritional intervention, the temptation has been to aim at improving nutritional status, all the while assuming a direct relationship between depleted nutritional status and adverse clinical outcomes, independent of the disease process. This model, although less complex and therefore less costly, is flawed as it leans towards the examination of surrogate endpoints rather than important primary outcomes. It also relies on an assumption that is potentially debatable. The failure to address clinical endpoints as primary outcomes in cachexia trials has lead to the current *status quo* in which intervention studies have had little impact on clinical practice.

1.12.2 Measures of nutritional status, functional status and quality of life

In the development of primary outcome variables for future cachexia intervention studies, it is useful to consider the relative advantages and disadvantages of the outcomes currently being used.

1.12.2.1 Weight loss and body composition

Nutritional outcomes that have been assessed in cachexia intervention trials have included a variety of anthropometric, biochemical and physiological variables [406, 408, 473-476]. Changes in body composition can be assessed with methods of varying complexity. Simple and inexpensive measures include body weight, BMI and bedside anthropometry using age-adjusted reference values (e.g. mid-arm muscle circumference [MAMC], triceps skinfold thickness [TSF]). Bio-impedance analysis (BIA) is a safe, quick and practical field-methodology that allows estimation of total body water (TBW), ECW, intracellular water (ICW), LBM and fat mass. However, the limitations of BIA mean that it is probably best for comparison of groups of patients rather than individuals. Dual-energy X-ray absorptiometry (DEXA), in-vitro neutron activation and tritiated water analysis, total body potassium and underwater weighing are all more specialised techniques.

Weight loss, as a simple measure of nutritional depletion, has been used with success as an entry criterion for various studies. Indeed, it is used in this respect throughout the Results section of this thesis. However, it can be argued that weight loss is inadequate to address fully the subtleties of cachexia. Despite being the most intuitive of all the outcomes currently employed, and perhaps the simplest to measure in a sensitive, objective manner, it remains difficult to determine exactly how much weight gain is required in the individual patient to be *clinically* significant, rather than *statistically* significant. Equally, total body weight represents the sum of a variety of different body compartments, and therefore documentation of a change in weight does not identify the specific nature of tissue

lost or gained. In other words, has the patient with weight loss actually experienced a depletion of skeletal muscle mass, or simply a loss of fat mass? Also, it is important to note that how weight loss is defined may influence the conclusions reached by clinical studies of cachexia. One such trial studied prospectively 8 weight-loss-related variables in patients with NSCLC [477]. The authors found that *total* weight loss was the best predictor of prognosis and that it was a superior measure to *rate* of weight loss [477].

The use of LBM as a primary end-point is perhaps superior to body weight alone as it reflects, at least to some degree, the functional protein mass in the body. It can be estimated either directly or indirectly by a variety of techniques, including anthropometry, BIA, isotope dilution or DEXA [478, 479]. However, as has been discussed previously in Chapter 1.2.2, as LBM is not a measure of a specific tissue mass, and also includes the mass of the ECW space, it is subject to some of the same criticisms as total body weight. As a patient becomes more oedematous, a change in LBM may be independent of the protein content of that mass. Furthermore, the functional significance of a change in lean tissue mass is still not established clearly.

Recently, cross-sectional imaging using MRI or CT has been utilised as a method of assessing patient muscularity. Usually, a single body compartment is assessed (e.g. mid-thigh muscle cross-sectional area; single-slice abdominal cross-sectional muscle area) as a surrogate measure of whole-body muscularity. In particular, L3 single-slice abdominal CT has been used with success in cancer patients, and has been capable of identifying obese patients with obvious muscle wasting (see

Chapter 1.2.2, p.38) [28]. Such 'sarcopenic obesity' was associated with poorer functional status compared with obese patients who did not have sarcopenia, and was an independent predictor of survival [30, 382].

There are a number of objective nutritional assessment tools available that can also be used to guide nutritional therapy [480]. The Mini-Nutritional Assessment (MNA) was developed initially as an inexpensive assessment tool to identify elderly persons ($\geq$ 65 years) at risk of malnutrition. It consists of 18 questions in 4 categories relating to simple anthropometry, general assessment, dietary assessment and subjective assessment. It has been shown to correlate with weight loss and CRP in patients with advanced cancer receiving palliative chemotherapy [481]. The Subjective Global Assessment (SGA) is a clinical tool that combines subjective and objective information from patient history and examination and then categorises patients according to nutritional status. It is effective in detecting cancer patients at risk of malnutrition [482]. The Prognostic Inflammatory Nutritional Index (PINI) was originally developed for the assessment of nutritional status and prognosis in critically ill patients [483]. In developing the tool, discrimant analysis of blood markers, including those pertaining to nutritional status, was used to select acute phase reactants (AGP, CRP) and visceral proteins (albumin, prealbumin). These were then combined in a formula to yield a score that was able to stratify critically ill patients according to their risk of complication and mortality. The authors suggested that it could be used to assess most pathological conditions, and it has been shown that the PINI is indeed highly abnormal in cancer patients [484, 485]. In such patients, high PINI scores correlated with elevated serum IL-6 levels [485].

1.12.2.2 Functional status

Poor PF in cachexia may relate to many factors including loss of body mass, reduced substrate supply (food intake) and reduced volitional effort (fatigue and depression), all of which can been related, at least in part, to the effects of systemic inflammation. The loss of physical reserve can lead to a devastating loss of independence. Furthermore, it may also exacerbate functional decline as inactivity is linked to muscle atrophy (see Chapter 1.8.2, p.92).

Clinically useful endpoints to assay changes in functional status have proved controversial. Functional status can be assessed subjectively or objectively. One basic subjective method that relies simply on questioning patients regarding their independence and ability to work and be active is physician-assessed PS (e.g. the KPS, WHO PS scores). These scores may be subject to bias but general agreement has been demonstrated between oncologist and patient-assessed WHO scores [486]. Palliative therapy (i.e. chemotherapy and radiotherapy) is influenced heavily by estimates of patient PS. PS tools have proven value as prognostic indicators in cancer patients [487] and as clinical trial inclusion criteria [488]. Attempts have also been made to validate accurately the scores as global markers of functional status [489]. However, despite growing interest in the functional assessment of cancer patients [490], it remains unclear exactly what PS scores measure and how they relate to true levels of patient activity. It has been suggested that PS suffers from being too narrow a tool [491], and that conventional PS scores may be less informative in older patients.

Objective assessment of a patient's PF can be performed using either simple techniques or more involved technologies that accurately measure a patient's PAL. Simple techniques involve direct measurement of muscle power (e.g. grip strength, treadmill testing and dynamic evaluation of leg extensor power). Leg strength correlates well with self-reported functional status in elderly women [492], and improvements in lower extremity strength are associated with gains in chair rise performance, gait speed, mobility and 'mobility confidence' in the elderly [493]. Hand-grip dynamometry is a readily measured tool, which is an accurate prognostic indicator in surgical patients [494]. The SFA combines a self-reported questionnaire, which collects information on symptoms and function, and a panel of nine physical tasks, including tying a belt, putting on a sock and walking tests [386]. However, all of these objective measures of PF simply measure one or a few facets of the patient's entire spectrum of activity. None capture the entire range of PF. Recently, objective assessment of spontaneous PA has been proposed as a more complete measure of PF and a more useful index of patient-orientated QoL [495]. This proposition is discussed later in the present section.

1.12.2.3 Quality of life

The palliative therapy of advanced cancer patients is often administered to improve symptoms, functional status and QoL. This is because objective tumour responses vary between only 20-70%, and often there is no survival advantage. For these reasons, research into QoL assessment has undergone a significant expansion in clinical oncology over recent years [490]. QoL is a multi-domain phenomenon and subject to observer variation; physician, patient and partner perception of a patient's

QoL can differ [496]. When asked what factors are important to QoL, patients group their responses into five distinct domains – their own state, the quality of their palliative care, their physical environment, relationships and outlook [497]. No commonly used single instrument at this time addresses all these issues. Instead, most tools use researcher-defined domains such as physical, functional, social and emotional well-being [498], and there is a concern that in so doing they may reflect the views of health professionals rather than patients. Indeed, the emphasis placed on health by many QoL tools may be questionable in patients with advanced cancer [499].

The measurement, analysis and interpretation of QoL data is challenging [500], and the complexity of the tools has resulted in selection bias secondary to patient drop out in some QoL studies [501]. Currently, the commonest tools for specific use in cancer patients include the European Organisation for Research and Treatment of Cancer (EORTC) QLQ-C30 questionnaire and the Functional Assessment of Chronic Illness Therapy [FACIT] questionnaires. The latter group of questionnaires have a wide range of tools for use in different forms of cancer and cancer therapy, including ones for the specific assessment of anorexia and cachexia therapy (Functional Assessment of Anorexia/Cachexia Therapy [FAACT]) and fatigue (FACIT-F). Unfortunately, different cancer QoL tools are not interchangeable and are unlikely to be directly comparable [502]. There have therefore been recent calls for an international consensus on how best to assess quality of life in oncology [503]. The EORTC QLQ-30, FACIT-F and FAACT questionnaires are employed later in this thesis (Chapter 11).

1.12.3 Current use of outcome measures

In the past ten years, there has been considerable interest in the potential therapeutic value of EPA for the treatment of cancer cachexia. A series of clinical trials have been conducted to assess the efficacy of EPA, and this body of literature provides a useful resource from which the current outcome measures reported in cachexia trials can be reviewed. Table 1.3 documents the main outcome variables measured in six of these studies [406-408, 473, 474, 504]. Immediately, it can be seen that there is no consensus as to which outcomes should actually be included in a trial of anti-cachexia therapy. Neither is there a standard methodology for assessing these outcomes. As can be seen in Table 1.3, although change in body weight was documented in all six studies, LBM was only measured in three. Food intake was measured in only three trials, and was assessed using either a 3-day diet diary or a linear analogue scale to document self-reported appetite. PF was assessed using either a self-reported tool as part of a QoL questionnaire, or physician-reported PS, using the KPS or WHO PS scales. It was documented variably by three of the trials, and it was not addressed at all by two major studies. Again, global QoL and survival duration from the initiation of treatment were documented by most of the studies, but not all. The obvious conclusions are that uncertainty surrounds both the methodology of assessing seemingly simple outcomes and the question of which variables should be examined in the first instance. The situation is equally confusing when we consider the use of *primary* outcome measures in these six studies. Weight was used as the primary outcome in two of the trials [407, 408]; the primary outcome measure was not stated explicitly in three of the trials (but appeared to be both weight and LBM in one trial [406]); and the primary outcome

	NUTRITIONAL STATUS		INTAKE		FUNCTIONAL STATUS		GLOBAL QoL	SURVIVAL
	Weight	LBM	Appetite VAS	FI	Self-report	PS		
Bayram 2009 * (n=52)	✓							
Fearon 2006 (n=518)	✓	✓			✓	✓	✓	✓
Burns 2004 (n=43)	✓				✓			✓
Jatoi 2004 (n=429)	✓		✓				✓	✓
Bruera 2003 (n=60)	✓	✓	✓	✓		✓	✓	
Fearon 2003 (n=200)	✓	✓		✓	✓	✓	✓	✓

Table 1.3 **Clinical outcome measures in EPA studies.**
There is a lack of agreement and consistency regarding the use of outcome measures in clinical trials of anti-cachexia therapies. A tick denotes use of an outcome. An underlined tick denotes the primary outcome (as either stated explicitly or assumed from the results of the publication). * paediatric study
FI: diet diary food intake; LBM = lean body mass; PS = performance score; QoL = quality of life; VAS = visual analogue score.

was appetite score in the last study [474]. Thus, clearly there is no agreement concerning the identity of the primary outcome measure in cachexia intervention trials either.

1.12.4 Importance of patient-centred outcome measures

The real value of any cachexia intervention can only be measured truly by assessing the day-to-day impact on the individual patient, whether that is an impact on QoL or patient independence. Over the past twenty years, the importance of such patient-centred outcomes has become evident. Studies have shown that cancer patients receiving palliative care are interested not only in the quantity of their remaining life, but also the quality. For example, in a survey of patients with advanced lung cancer, only 22% of patients chose palliative chemotherapy, in preference to supportive care alone, to benefit from the associated 3-month survival advantage [505]. In contrast, 68% of patients chose chemotherapy when it substantially reduced adverse symptoms without prolonging life. However, QoL is a very complex issue to address. It is a multidimensional construct that includes clinical, psychological, physical, cognitive, emotional, spiritual and social domains. It is therefore best-measured using tools that assess multiple domains of patient well-being [506-508], as well as common symptoms of cancer and its treatment (e.g. pain, nausea, fatigue). It is generally accepted that QoL is a subjective phenomenon, which is best assessed by the individual patient. Indeed, studies have shown that considerable disparities exist between concurrent ratings of QoL made by patients and their physicians [509, 510]. Various attempts have been made to develop a crude assessment of overall QoL, and these are generally known as global QoL

measurements [511]. However, when determining the impact of a nutritional intervention on QoL, rather than measuring global QoL (which is likely to be influenced by many other factors independent of nutritional status), it might be more relevant to assay the intensity of a specific symptom that is readily influenced by nutritional status. The FAACT and FACIT-F tools, which target anorexia-cachexia and fatigue respectively, are appropriate examples of this strategy. However, in particular, the PF of a patient is one such proximal index of QoL that might be useful in application to cachexia intervention trials [495].

1.12.5 Timing of the primary outcome measure

In general, cachexia intervention trials study patients over a period of weeks, looking for a change in either body composition, functional status or a combination of both. However, there is no agreement regarding how long any interventional study should be. To detect a measurable change in body composition usually requires a trial lasting 6-8 weeks. In the six EPA trials examined above, the duration of the intervention ranged from 2 weeks to 6 months [406-408, 473, 474, 504]. Assessment of functional status may have an advantage over nutritional status, as it tends to improve ahead of changes in body composition and therefore may be demonstrable over a shorter timescale (e.g. days). However, no matter which variable is selected as primary outcome measure, cachectic cancer patients are generally so unwell when entered into intervention trials that less than 50% are still assessable at 8 weeks from treatment initiation [406]. This fact suggests that there is a probable selective attrition of the patient population, a problem that has not been adequately addressed by refinement of trial methodology.

1.12.6 Physical activity as a patient-centred outcome measure

When we consider all of the potential difficulties in the measurement of current outcomes in cachexia trials, the objective assessment of patient PA (as a global measure of complete PF) offers a viable and advantageous alternative. PF is an important domain of QoL that may be strongly influenced by nutritional status. Furthermore, it is a direct product of skeletal muscle action, the major tissue type depleted by cancer cachexia. Thus, objective PA is a clinical biomarker of both nutritional and functional status, and could be utilised as a primary outcome measure.

To improve PA in cancer cachexia, there are a number of areas that may be targeted (Figure 1.19). LBM (the patient's "*engine*") may be optimised either by size and/or by the efficiency of its function. Food intake (the patient's "*fuel*") may be optimised by increasing the total supply of macronutrients (more calories) and/or improving the energy quality or density of the food. Finally, medical, nursing or physiotherapy staff may attempt to motivate the cachectic patient (the "*driver*") to mobilise, or instruct the patient on how to utilise their limited energy reserves more efficiently. Crucially, it has been proven that PA can be improved by anti-cachexia interventions (see Chapter 1.8.2, p.92) [406]. Moses and co-workers demonstrated that the baseline PAL (ratio of TEE/REE) of cachectic pancreatic cancer patients was very low (with patients spending prolonged periods at rest or in bed) [406]. Patients were randomised to receive either an energy and protein dense oral nutritional supplement, or the same supplement enriched with a pharmacological dose of EPA. The combined regimen was designed not only to improve patients'

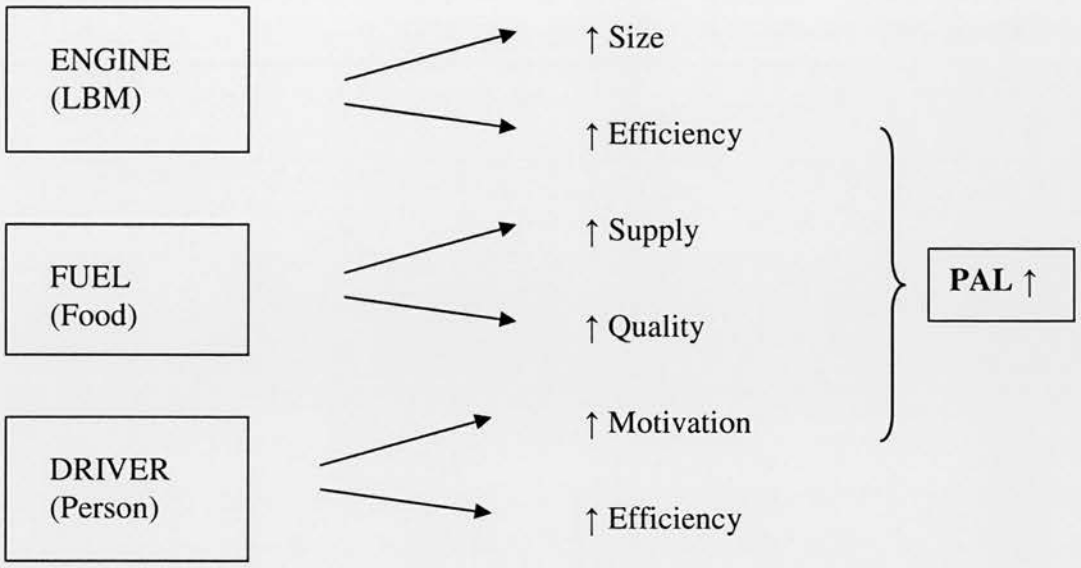

Figure 1.19 **Improving physical activity in cancer cachexia.**
In order to improve PA in cachectic cancer patients, LBM (the patient's "*engine*") may be maximised either by size and/or by the efficiency of its function; food intake (the patient's "*fuel*") may be maximised by increasing the total supply of macronutrients (more calories) and/or improving the energy quality or density of the food; and the cachectic patient (the "*driver*") may be encouraged to mobilise, or instructed on how best to utilise their limited energy reserves more efficiently.
LBM = lean body mass; PAL = physical activity level

food intake but also to address some of the underlying metabolic abnormalities that contribute to the syndrome of cachexia (e.g. systemic inflammation). Patients receiving the conventional supplement did not demonstrate increased PAL. However, PAL increased significantly in those patients receiving the combined regimen, to values commensurate for sedentary office workers [512]. This is one of the few randomised studies that has used objective methodology and shown that a nutritional intervention can improve PF in advanced cancer patients. Furthermore, there is evidence emerging that PA does indeed reflect and correlate with QoL scores in cancer patients [380, 513].

However, despite the primacy of patient PA as an outcome measure, the gold-standard methodologies for objectively assessing PA are expensive, time-consuming, work-intensive and not patient-friendly. The classical methodology for determining PAL involves combined measurement of TEE by the doubly labelled water (DLW) stable isotope technique and REE by indirect calorimetry (Figure 1.20). PAL represents estimated energy requirements as a multiple of the basal metabolic rate (BMR) and is derived from the ratio of TEE to REE. PA is a significant and variable component of TEE in free-living individuals. Although REE may be increased in some wasted cancer patients, TEE may actually fall suggesting that they modulate energy demand by reducing PA [406].

In recent years, the development of accelerometer-based PA meters now means that there are viable, cost-effective, patient-friendly and reusable technologies to assess patient PA in the free-living, outpatient environment. In particular, the activPAL™

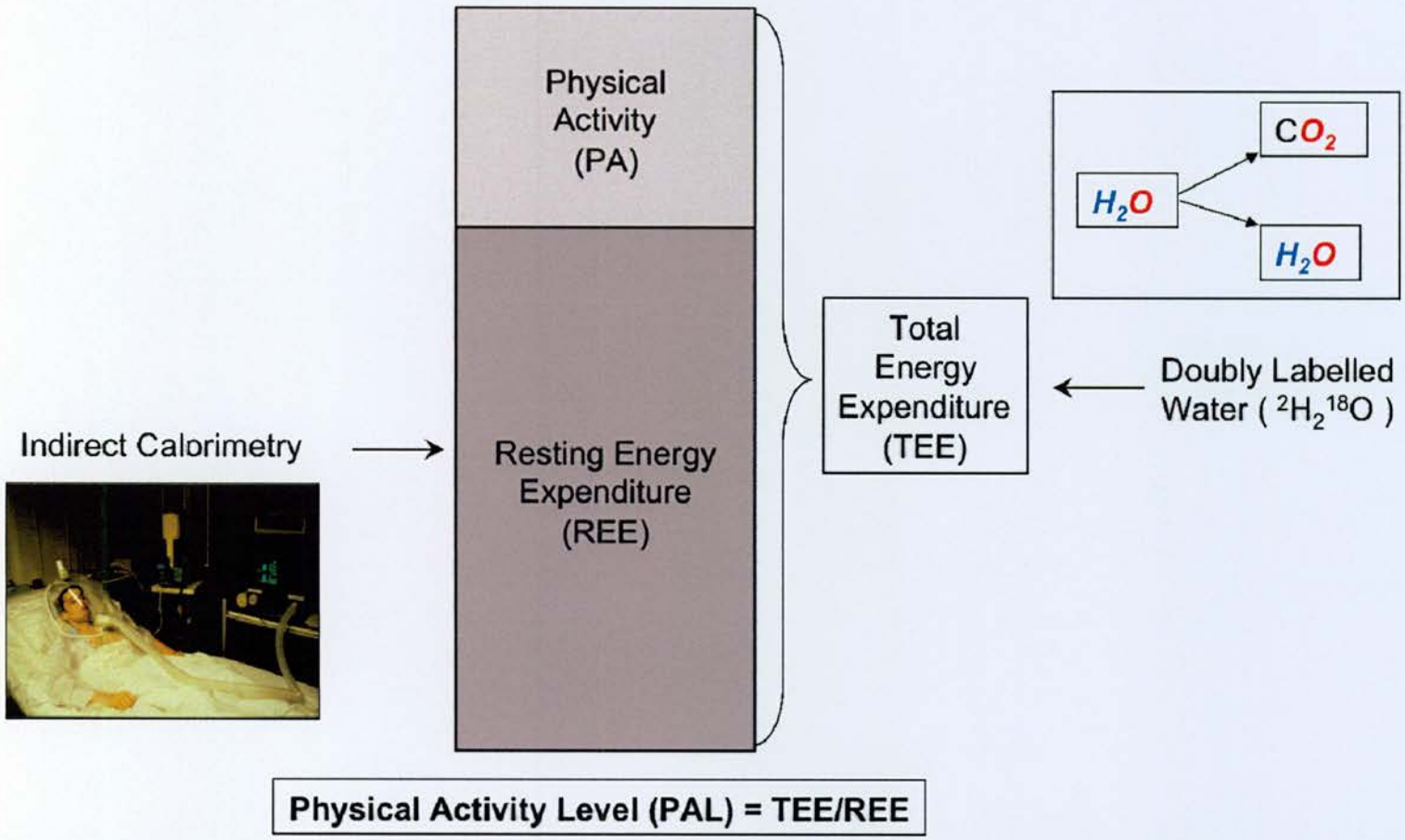

Figure 1.20 **Techniques of objective measurement of physical activity I: doubly labelled water and indirect calorimetry.** The gold-standard methodology for the objective assessment of PA is to use doubly labelled water to assess total energy expenditure (TEE) and indirect calorimetry to measure resting energy expenditure (REE). The ratio of TEE/REE represents the Physical Activity Level (PAL). However, this technique is very expensive, time consuming and impractical to use in a regular outpatient setting.

meter (Figure 1.21) has been used to objectively measure PA in a range of patient cohorts, including cachectic cancer patients (median weight loss 9%) undergoing palliative chemotherapy [380] (Figure 1.22). This latter study demonstrated that estimated TEE was 8% lower, time spent upright was approximately 2 hours/day less, and step count was 43% lower than that of age-matched, healthy controls [380]. The activPAL™ meter is studied later in this thesis (Chapters 10 and 11). Firstly, its ability to measure EE is validated against the DLW technique. Secondly, it is employed in a cohort of patients undergoing palliative chemotherapy in order to develop further objective PA as a clinical biomarker of nutritional status, functional status and QoL in advanced cancer and cachexia.

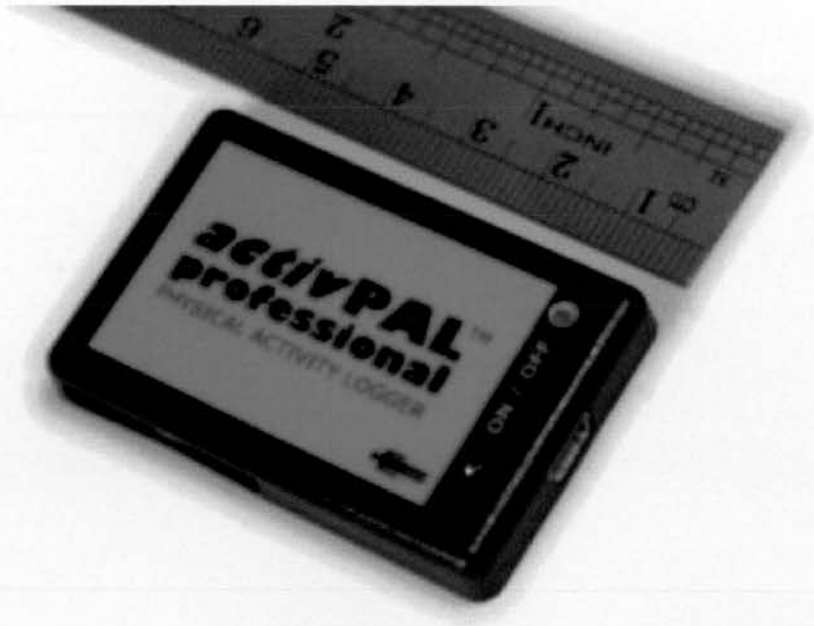

Figure 1.21 **Techniques of objective measurement of physical activity II: activPAL™ meter.**
The activPAL™ meter is a lightweight, accelerometer-based device that is secured to the patient's anterior thigh, and which measures objectively a number of PA-related variables. For a full description of the meter, see Methods Chapter 2.10.1, p.190.

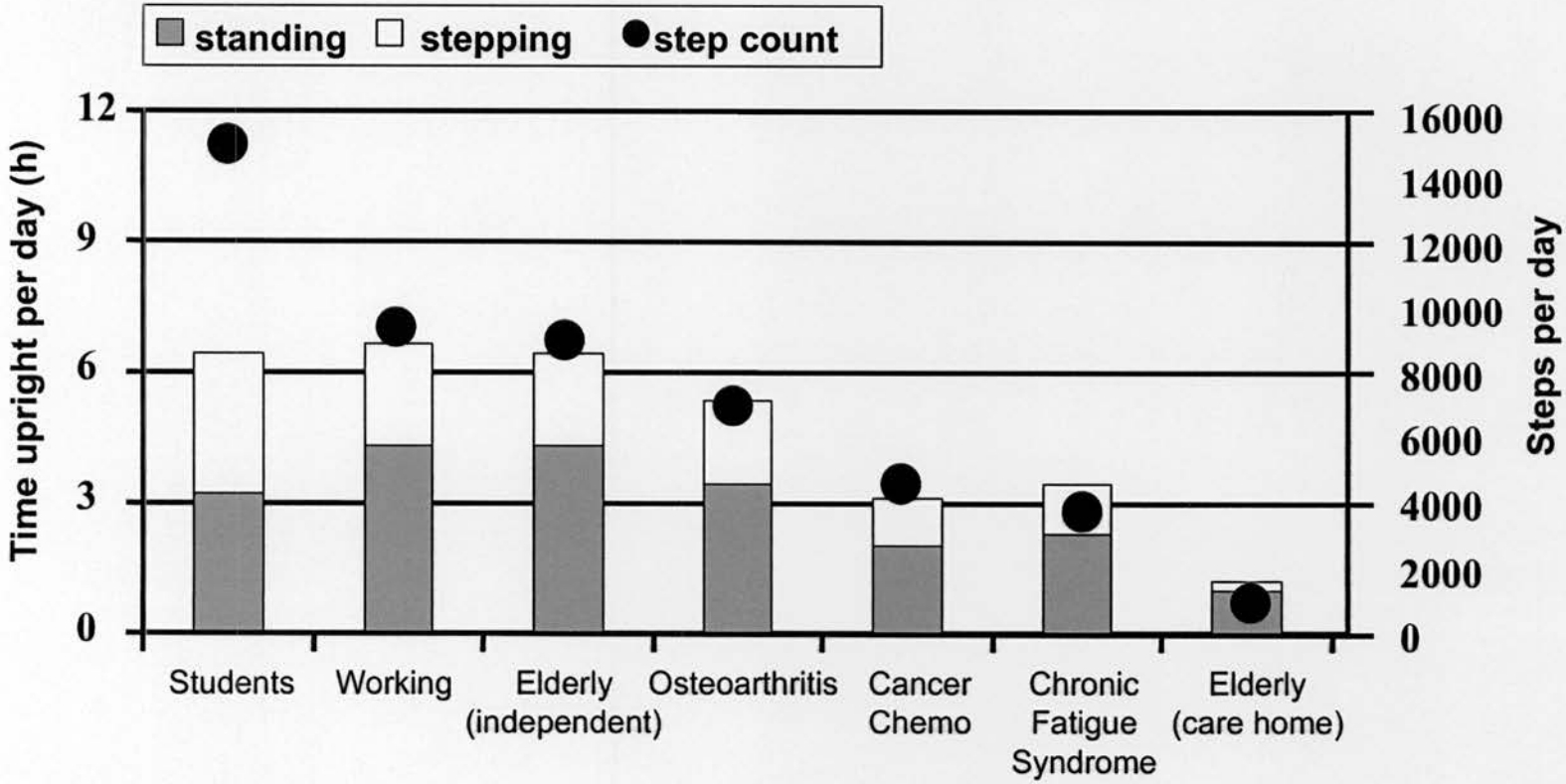

Figure 1.22 **ActivPAL™-derived assessments of physical activity in a range of different patient cohorts.**
The activPAL™ meter is able to provide information on a range of different physical activity-related outcome measures including time spent standing and time spent stepping (which cumulatively add up to time spent upright), and step count. Studies such as this firmly establish the impact of cancer cachexia and palliative chemotherapy on physical functioning. In this case, the studied cohort of cachectic patients demonstrated worse physical functioning than patients with severe lower limb osteoarthritis. Adapted from Douglas Maxwell of PAL Technologies Ltd.

meter (Figure 1.21) has been used to objectively measure PA in a range of patient cohorts, including cachectic cancer patients (median weight loss 9%) undergoing palliative chemotherapy [380] (Figure 1.22). This latter study demonstrated that estimated TEE was 8% lower, time spent upright was approximately 2 hours/day less, and step count was 43% lower than that of age-matched, healthy controls [380]. The activPAL™ meter is studied later in this thesis (Chapters 10 and 11). Firstly, its ability to measure EE is validated against the DLW technique. Secondly, it is employed in a cohort of patients undergoing palliative chemotherapy in order to develop further objective PA as a clinical biomarker of nutritional status, functional status and QoL in advanced cancer and cachexia.

In this section, following a critical appraisal of the current *status quo* of clinical trial methodology, the objective assessment of PA has been proposed as a novel outcome measure for use in studies of anti-cachexia therapeutic interventions. This section represents the last of the introductory sections that summarise cancer cachexia. The following section details the objectives for the present thesis.

1.13 Thesis objectives

This chapter has attempted to provide a full summary of the current knowledge base of cancer cachexia, including some of the remaining unanswered questions and controversies. In particular, this chapter has stressed that skeletal muscle is the key tissue type affected by cancer cachexia, and that wasting of this tissue is the most significant determinant of patient function and outcome. Furthermore, it has highlighted that there is a pressing need for biomarkers of cachexia (either

biological or clinical) for effective clinical trial design. These biomarkers could act as inclusion criteria, outcome measures or both. Lastly, it has been emphasised that the majority of mechanistic studies have been carried out in animal models and *in vitro* systems, and that there remains a paucity of human data. Therefore, this thesis is aimed at the study of mediators, mechanisms and biomarkers of skeletal muscle wasting and function in cancer cachexia, using human cell lines, human samples and cancer patients as the investigative models of choice. A number of diverse studies in this field have been carried out, with the main objectives appearing below:

1. Quantify the expression of dermcidin mRNA (as a proxy of PIF expression) in primary upper GI cancer and prostate cancer tissue (see Chapter 1.6.1, p.69);

2. determine the expression of dermcidin mRNA (as a proxy of PIF expression) in a range of human cancer cell lines (see Chapter 1.6.1, p.69);

3. analyse the sequence of dermcidin cDNA from human cancer cell lines in order to investigate the potential for PIF glycosylation (see Chapter 1.6.1, p.69);

4. analyse the effect of serum sex steroids and gonadotropins on nutritional status, systemic inflammation and survival in cancer patients (see Chapter 1.5.1.1, p.56);

5. analyse the effect of plasma MIC-1 on nutritional status, food intake, systemic inflammation and survival in cancer patients (see Chapter 1.7.1.5, p.87);

6. determine the presence of DGC abnormalities in muscle from cancer patients and relate such abnormalities to nutritional status, systemic inflammation, and survival (see Chapter 1.9.1.4, p.106);

7. determine the expression of phosphorylated and non-phosphorylated forms of PKR and eIF2α in muscle from cancer patients and relate such expression to nutritional status and survival (see Chapter 1.9.1.2, p.104);

8. investigate the presence of biomarkers of skeletal muscle wasting in urine from cancer patients (see Chapter 1.9.2, p.108);

9. validate estimates of EE measured by the activPAL™ PA meter using a DLW stable isotope technique (see Chapter 1.12.6, p.157);

10. measure objective PA in palliative cancer patients undergoing chemotherapy, and relate such measurements to nutritional status, mood and QoL.

Chapter 2 – Patients, Materials and Methods

2.1 Patients and healthy controls

For all studies, patients with upper GI cancer (OGC and pancreatic cancer) were recruited from the Surgical Department of the Royal Infirmary of Edinburgh and the Oncology Department of the Western General Hospital, Edinburgh. Staging was carried out according to the American Joint Committee on Cancer (AJCC)/Union Internationale Contre le Cancer (UICC) systems [514]. Blood, tissue and urine samples were collected from these individuals. If patients were undergoing cancer surgery with or without curative intent, samples of skeletal muscle and tumour tissue were also collected (see Chapters 2.4, p.173). Local ethics committees approved the protocols, and written informed consent was obtained from all patients. Procedures were in accordance with the International Committee for Harmonization, Good Clinical Practice, and the Helsinki Declaration.

Samples of blood, urine and/or skeletal muscle were also obtained from patients undergoing minor surgical procedures for benign, non-inflammatory conditions (e.g. hernia repair), or laboratory/hospital staff, for use as healthy controls. Samples of oesophago-gastric control tissue were taken from patients having normal upper GI endoscopies (see Chapter 2.4.2, p.174).

Specific circumstances and inclusion/exclusion criteria of certain patient and control cohorts are described in detail in the Results chapters.

2.2 Nutritional Assessment

2.2.1 Weight and height

At recruitment, pre-illness stable weight and duration of weight loss were self-reported by cancer patients, in order to calculate percentage weight loss [515]. Patients were weighed on spring balance scales without shoes and wearing light clothing. With the exception of the sex steroid/gonadotropin study (Chapter 3) for which weight loss >5% of pre-illness stable weight over the previous 6 months was one of the initial inclusion criteria for the original randomised trial, cachexia was defined as ≥10% weight loss. Height was measured using a wall-mounted stadiometer with the patient standing erect without shoes.

2.2.2 Lean body mass

Body composition, including LBM (as an estimate of skeletal muscle mass) and body water, was assessed using BIA. In the sex steroid/gonadotropin study (Chapter 3), a Xitron Hydra multiple-frequency bio-impedance analyser (Xitron Technologies, San Diego, USA) was used, whereas in the activPAL™ studies (Chapters 10 to 12), a Bodystat Quadscan 4000 multiple-frequency bio-impedance analyser (Bodystat Ltd, Isle of Man, UK) was used. Such techniques have been validated previously in similar patients [516, 517], and have been used in comparative studies of large patient groups [406, 407].

2.2.3 Anthropometry

Mid-arm circumference (MAC) was measured (in cms) at the mid-point between the acromion and olecranon processes in the non-dominant arm. TSF, as a measure

of adiposity, was measured (in mms) with Harpenden skin callipers (Holtain, Crymych, UK). MAMC, as a measure of muscularity, was calculated according to the formula: MAMC = MAC-[π x TSF/10].

2.2.4 Grip strength

Maximal grip strength was assessed by a hand-held spring-loaded dynamometer (Takei, Nigata, Japan). The maximum reading from 3 attempts using the dominant hand was recorded.

2.2.5 Performance status

KPS and WHO PS scores (see Appendix II, p.484) were documented by the recruiting physician.

2.2.6 Assessment of dysphagia and dietary intake

Dysphagia score (normal swallowing = 0; dysphagia to solids = 1; dysphagia to softened foods = 2; dysphagia to liquids = 3; and total dysphagia = 4) and subjective diet score (normal dietary intake = 1; reduced dietary intake = 2; and poor dietary intake = 3) were used as surrogate measures of food intake.

2.3 Blood tests

2.3.1 Plasma and serum separation

For plasma separation, venous blood collected in lithium heparin tubes was centrifuged at 1,500rpm at 4°C for 5min. For serum separation, clotted blood was centrifuged at 1,500rpm for 30min. Both plasma and serum samples were stored as

1mL aliquots at -80°C until analysis.

2.3.2 Analysis of circulating CRP concentration

For the sex steroid/gonadotropin study, serum CRP concentration was measured by enzyme-linked immunosorbent assay (ELISA) (Dako, High Wycombe, UK), according to the manufacturer's instructions [518]. The limit of detection was 1mg/L. Coefficient of variation (CV) was <9% across the concentration range studied. For all other studies, plasma CRP was assayed using automated methods on an Olympus AU2700 analyser (Olympus Diagnostica GmbH [Irish Branch], Lismeehan, Ireland), in the Department of Clinical Chemistry, Royal Infirmary of Edinburgh (fully accredited by Clinical Pathology Accreditation [UK] Ltd).[1] Appropriate internal quality controls (IQC) were included, with CVs typically 3.4% at concentrations <15mg/L and 1.6% at 80mg/L. Presence of systemic inflammation/APPR was defined by a plasma/serum CRP ≥10mg/L.

2.3.3 Analysis of circulating albumin concentration

Plasma/serum albumin concentration was assayed using automated methods on an Olympus AU640 analyser (Olympus Diagnostica GmbH [Irish Branch], Lismeehan, Ireland), in the Department of Clinical Chemistry, Royal Infirmary of Edinburgh. Appropriate IQC were included, with CVs typically <3.0% at all concentrations[1]. By combining the results of both plasma CRP and albumin concentrations (Chapter 4), the mGPS could be calculated as follows: patients with plasma CRP >10mg/L were allocated a mGPS of 1 or 2 depending on the absence

[1]I acknowledge Dr. Catharine Sturgeon of the Department of Clinical Chemistry, Royal Infirmary of Edinburgh, who performed these analyses.

or presence of hypoalbuminaemia (<35g/L), whereas patients with plasma CRP ≤10mg/L were allocated a mGPS of 0.

2.3.4 Analysis of serum gonadotropin, sex steroid and CA19.9 concentrations

A single blood sample was drawn in the morning between the hours of 0930 and 1200. Total testosterone (TT), oestradiol, luteinising hormone (LH), follicle stimulating hormone (FSH), sex hormone binding globulin (SHBG), albumin and carbohydrate antigen 19-9 (CA19-9) were assayed using automated methods (Bayer Immuno-1 for all analytes except SHBG [DPC Immulite]) on an Olympus AU640 analyser in the Department of Clinical Chemistry, Royal Infirmary of Edinburgh[1]. Appropriate IQC were included, with between-run CVs typically <8%. Male oestradiol concentrations and female control TT concentrations were not available. Control albumin concentrations were not measured but were assumed to be 42.5g/L, the median of the reference range. Calculated free testosterone (cFT) was derived from TT, albumin and SHBG using the Vermeulen formula [519]:

$$cFT = \frac{TT-n-S+\sqrt{[(n+S-TT)^2 \times 4nTT]}}{2n}$$

where TT= total testosterone

$n = (0.5217 \times A) + 1$

S = SHBG in nmol/L

A = albumin in g/L

Male hypogonadism was defined either as TT <10nmol/L or normal TT (10-30nmol/L) with raised LH (>9.0U/L), known henceforth as 'TT definition', or as cFT <0.245nmol/L, known henceforth as 'cFT definition' [519]. In female subjects, elevated oestradiol was defined as >150pmol/L, the upper limit of normal for postmenopausal women.

2.3.5 Analysis of serum IL-6 and sTNFR75 concentrations

Serum IL-6 and soluble TNF receptor 75 (sTNFR75) concentrations were measured by ELISA (Quantikine, R&D Systems, Abingdon, UK), according to the manufacturer's instructions[2]. Limits of detection were 0.5 pg/mL, and CVs were <9% across the concentration ranges studied.

2.3.6 Analysis of plasma MIC-1 concentration

Samples were examined in duplicate using a well-established sandwich ELISA as previously described [520][3]. Briefly, 96-well Maxisorp ELISA plates were coated with mAb 26G6H6 supernatant diluted 1:5 (final immunoglobulin [Ig] concentration was approximately 20ng/mL) in coating buffer at 4°C for 24h. ELISA plates were then washed three times with 300μL/well of wash buffer. Non-specific binding was blocked with 250μL/well of 1% (wt/vol) bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 2h at 37°C. Recombinant human MIC-1 (rhMIC-1) standards and plasma samples in Ab Dil were then added to the plates (100μL/well) and incubated for 1h at 37°C. The plates were washed three times,

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[2]I acknowledge Mr. Alastair Moses who performed these analyses.
[3]I acknowledge Dr. David Brown, Mr. Mark Hunter and Prof. Sam Breit of the Centre for Immunology, St. Vincent's Hospital and University of New South Wales, Sydney, Australia, who perfomed these analyses.

followed by the addition of 100μL/well of the sheep polyclonal antibody (pAb) 233B3-P diluted 1:5000 in Ab Dil and incubated for 1h at 37°C. ELISA plates were then washed three times, and 100μL/well of biotinylated donkey antisheep IgG diluted to 1:5000 in Ab dil was added and incubated for 1h at 37°C. The plates were then developed and the MIC-1 plasma concentration was determined by reference to a standard curve constructed using rhMIC-1 as the standard. All samples had duplicate values with a CV <10%. Assay performance was monitored additionally using standard diagnostic laboratory procedures.

2.3.7 Analysis of plasma creatinine and creatine kinase concentrations

Plasma creatinine concentration was measured using a kinetic Jaffe technique on an Olympus AU2700 analyser (Olympus UK Ltd, Watford, UK) (normal defined as <120μmol/L)[1]. Plasma creatine kinase (CK) concentration was measured using a CK- N-acetyl-L-cysteine (NAC) (International Federation of Clinical Chemistry and Laboratory Medicine: IFCC) technique, also on an Olympus AU2700 analyser (normal defined as <170U/L in males and <135U/L in females)[1]. Within and between-run CVs were <2.0% at all concentrations for both tests.

2.4 Tissue biopsies

2.4.1 Skeletal muscle biopsies

Samples of rectus abdominis muscle were obtained without the use of diathermy from the edge of the abdominal wounds of patients undergoing cancer surgery with or without curative intent or controls undergoing minor surgical procedures for

benign, non-inflammatory conditions e.g. hernia repair. Samples were taken within 20 minutes of induction of general anaesthesia. Samples were frozen immediately in liquid nitrogen using liquid nitrogen-resistant tubes (Corning BV, Netherlands), and then stored at -80°C until analysis.

2.4.2 Oesophageal and pancreatic tissue biopsies

Samples of tumour tissue were dissected from the resected cancer specimens by a Consultant Pathologist within 20 minutes of the staple guns being fired and the blood supply of the resection specimen being interrupted. Samples were frozen immediately in liquid nitrogen using liquid nitrogen-resistant tubes (Corning BV, Netherlands), and then stored at -80°C until analysis. A Consultant Pathologist analysed representative tissue sections to confirm the presence of malignant cells in tumour samples and the absence of malignant cells within the benign samples. Control gastric and oesophageal biopsies were collected from healthy volunteers undergoing negative endoscopic investigation of GI symptoms[4]. For patients undergoing pancreatectomy, samples of tumour and normal tissue were removed from fresh surgical specimens by a Consultant Pathologist. For patients undergoing palliative bypasses for pancreatic cancer, core biopsies of tumour tissue were taken intraoperatively[5].

2.4.3 Prostatic tissue biopsies

Samples of malignant and benign human prostate tissue were obtained from the Partners in Cancer Research Tissue Bank, held in the Department of

[4]I acknowledge Mr. Christopher Deans who collected these control biopsies.
[5]I acknowledge Mr. Alastair Lowrie who collected the pancreatic tumour biopsies.

Histopathology at the Norfolk & Norwich University Hospital. Detailed procedures for obtaining informed patient consent, tissue acquisition, and histopathological and molecular quality control and validation have been described [521][6]. Samples of malignant prostate tissue were collected from patients undergoing radical prostatectomy or channel transurethral resection of the prostate (TURP), and non-malignant samples were obtained from patients undergoing radical cystoprostatectomy for transitional cell carcinoma of the bladder or TURP for benign prostatic hypertrophy (BPH).

2.5 Urine samples

2.5.1 Urine collection

Morning urine samples were taken and stored at -20°C until analysis. First urine samples of the day were not used in order to avoid urine that may have remained in the bladder overnight.

2.5.2 Urinary protein precipitation

Following centrifugation to remove debris, proteins were precipitated from 20mL urine by incubation at -20°C for 2h with 40mL cold acetone containing 20mM dithiothreitol (DTT). Samples were centrifuged and the protein pellet was re-dissolved in 3mL PBS/3mL rehydration buffer (8mol/L urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), 20mM DTT). A 200µL aliquot was centrifuged and the supernatant assayed for protein concentration using the Bradford technique (BioRad Protein Assay kit, USA).

[6]I acknowledge Mr. Anthony Riddick and Mr. Grant Stewart who forged this collaboration and collected several of these samples.

Absorbance was read at 630nm (MRX II spectrophotometer, Dynex Technologies) compared to a standard reference curve created using a 1:1:1 dilution of bovine-γ-globulin protein standard with PBS and re-hydration buffer.

2.6 Cell culture

2.6.1 Cell lines

The following immortalised human cancer cell lines were obtained from the European Collection of Cell Cultures (ECACC), Porton Down, UK: hormone-sensitive prostate cancer cell line LNCaP; hormone-insensitive prostate cancer cell lines PC-3 and DU145; human hepatocellular carcinoma cell lines Huh-7 and Hep G2; and human pancreatic adenocarcinoma cell lines PANC-1, CFPAC and MIA-Pa-Ca-2 (Table 2.1). Hormone-sensitive prostate cell line PC-3M was kindly donated by Dr C Pettaway, University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA, whereas human malignant melanoma cell line G-361 was kindly donated by Professor Michael Tisdale, Aston University, UK. Prostate cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Paisley, UK). Melanoma G-361 cells were cultured in McCoy's 5A medium (Invitrogen). Hepatocellular carcinoma and pancreatic cancer cell lines were cultured in Dulbecco's Modified Eagle Medium (Invitrogen). Media were supplemented with 10% foetal calf serum (FCS), 50u/mL penicillin, 50μg/mL streptomycin and 2mM glutamine (all from Invitrogen, Paisley, UK). Cells were grown in 75cm^2 flasks (Corning BV, Schipol-Rijk, The Netherlands).

Tissue Type of Origin	Cell Lines
Hormone-sensitive prostate cancer	LNCaP PC-3M
Hormone-insensitive prostate cancer	PC-3 DU145
Hepatocellular cancer	Huh-7 Hep G2
Pancreatic adenocarcinoma	PANC-1 CFPAC MIA-Pa-Ca-2
Malignant melanoma	G361

Table 2.1 **Cell lines used in experimental work.**
The above listed cell lines were utilised in the investigations pertaining to PIF within this thesis. These cell lines were chosen as they either represent the tissue type in which dermcidin expression was first described (i.e. prostate cancer – see Chapter 1.6.1, p.69) or they represent the tissue type most commonly associated with cancer cachexia (i.e. upper gastrointestinal cancer types - see Chapter 1.3, p.41).

2.6.2 Transfection of cells with DCD cDNA for use as a positive control

Wang *et al* demonstrated that the PC-3M cell line does not express DCD mRNA [197] and it was thus chosen as a suitable cell line to be transfected stably with a pcDNA3.1+DCD vector, in order to create a robust positive control for DCD real-time PCR. The pcDNA3.1+ mammalian expression vector (Invitrogen, Paisley, UK) had full length DCD cDNA directionally cloned into the multiple cloning site using the EcoRI and BamHI restriction enzymes (Promega, Southampton, UK). pcDNA3.1+DCD vector was transfected stably into PC-3M cells using FuGENE-6 (Roche Applied Science, Lewes, UK)[7]. Geneticin selection antibiotic (Invitrogen, Paisley, UK) (600μg/mL) was used to select transfected cells and eventually create stable transfectants. Sham transfected PC-3M cells were created using an empty pcDNA3.1 vector as a control. Geneticin (300μg/mL) was used to supplement medium to maintain the transfected cells.

2.7 Polymerase chain reaction

2.7.1 RNA isolation

For RNA preparation from intact upper GI and pancreatic tissue samples, a rotor-stator homogeniser was used for homogenisation. RNA was then extracted using an RNAeasy kit (Qiagen, Crawley, West Susses, UK) according to the manufacturer's instructions. Certified ribonuclease (RNAse)-free pipette tips and microcentrifuge tubes were used at all times. RNA was resuspended in nuclease free water and

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[7]I acknowledge Mr. Grant Stewart who performed this transfection.

concentrations determined by spectrophotometry using a GeneQuant pro RNA/DNA calculator (Amersham Pharmacia Biotech, Bucks, UK). For RNA preparation from cell lines, RNA was extracted using the TRizol method according to the manufacturer's instructions (Invitrogen, Paisley, UK). Following resuspension, RNA concentrations were determined by spectrophotometry using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, UK).

2.7.2 Reverse transcription polymerase chain reaction (RT-PCR)

One mg of total RNA was used for reverse transcription (RT). RNA was first deoxyribonuclease (DNAse)-I-treated using RQ1 DNAse (Promega, Southampton, UK) to ensure no contamination. Reverse transcription was performed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Southampton, UK) according to the manufacturer's instructions. cDNA was stored at -20°C until used.

Two different sets of primers (named *PIF* and *HCAP*) were used with individualised polymerase chain reaction (PCR) programmes to identify DCD cDNA. *PIF* primers were designed using the Primer3 primer design programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and used with *β actin* housekeeping primers. All primers were manufactured by Sigma, Irvine, UK.

PIF forward primer: CCTCTTCCTGACAGCTCTGG

PIF reverse primer: CTGGATCTCTGCTTCCTTGG

β actin forward primer: ACTGACTACCTCATGAAGAT

β actin reverse primer: CGTCATACTCCTGCTTGCTGAT

HCAP forward primer: ACTCTCCTCTTCCTGACAGCTCTGG

HCAP reverse primer: CTGCTGCTCCTGGGTATCATTTCTC

MIC forward primer: CACGTCATCCAGCAGAGAATGGAAAGTC

MIC reverse primer: TGACCAAGATGTTGATGTTGGATAAGAG

Figure 2.1 shows the primer targets on the DCD nucleotide sequence.

Each reaction was performed in 25μL and contained the equivalent of 1μL of reverse transcribed RNA, 1μM each of the forward and reverse primer, 0.2mM deoxyribonucleotide triphosphate (dNTP) (Invitrogen, Paisley, UK), 2.5mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Promega, Southampton, UK). Conditions for both the *PIF* and *β actin* PCR reactions were: 94°C for 5min, then 35 cycles, consisting of 94°C for 1min, 56°C for 1min and 72°C for 1min, followed by 72°C for 10min. *HCAP* primers designed by Wang *et al* (which encompass the PIF nucleotide sequence) [197], were used with β_2-microglobulin (*MIC*) PCR control for RNA quality and RT performance. Conditions for both the *HCAP* and *MIC* PCR reactions were: 80°C for 3min, then 35 cycles, consisting of 95°C for 5s and 69°C for 1min, followed by 72°C for 7min. *HCAP* (361 base pairs [bp]) and *MIC* (555bp) PCR products separated on a 1.6% agarose gels (agarose (Flowgen Bioscience, Nottingham, UK) dissolved in 1X Tris-acetate-EDTA (TAE; Appendix I, p.483) with 5μl ethidium bromide run in 1XTAE. *PIF* (174bp) and *β actin* (535bp) PCR products were separted on 6% acrylamide gels run in 1X Tris-borate-EDTA (TBE; Appendix I, p.483), for enhanced resolution. A 50-1000bp PCR Marker (Promega, Southampton, UK) was used as a DNA size marker.

gaccctagatcccaagatctccaaggatttggtggcatacccactccagcacacagaagcATGaggt

tcatg*actctcctcttcctgacagctctgg*caggagccctggtctgtgcct↑**atgatccagaggccgcct**

ctgccccaggatcggggaacc↑cttgccatgaagcatcagcagctcaaaaggaaaatgcagg

tgaagacccagggttagccagacaggcaccaaagccaaggaagcagagatccagccttctgg↑aa

aaaggcctagacggagcaaaaaaagctgtgggggactcggaaaactaggaaaagatgcagtcga

agatctagaaagcgtgggtaaagg↑agccgtccatgacgttaaagacgtccttgactcagtactaTAG

ctgtaaggagaagct*gagaaatgatacccaggagcagcag*gctttacgttttcagcctaaaacct

Figure 2.1 ***DCD* gene nucleotide sequence with marked targets for *PIF* and *HCAP* PCR primers.**

PIF-core peptide nucleotide sequence in bold. ↑ denotes site of an intron. *PIF* primer targets singly underlined. *HCAP* primer targets in italics. Putative O-glycosylation sites doubly underlined, whereas putative N-glycosylation sites dotted underlined. Start and stop codons are shown in upper case.

2.7.3 Real-time polymerase chain reaction

cDNA preparation and real-time PCR were performed as previously described [522, 523][8]. Briefly, 1μg of total RNA was reverse transcribed using 2μg random hexamers (Amersham, Bucks, UK) and Superscript II reverse transcriptase (Life Technologies, Paisley, UK) according to the manufacturer's instructions. cDNA was stored at -20°C until used.

For PCR reactions, specific primers and fluorogenic probes for DCD were designed using Primer Express 1.0 software (Applied Biosystems, Warrington, UK) and synthesised by Applied Biosystems. Figure 2.2 shows the primer and probe targets on the *DCD* nucleotide sequence, which partially overlaps with the nucleotide sequence for the PIF-CP.

DCD real-time forward primer: CAAAAGGAAAATGCAGGTGAAGA
DCD real-time reverse primer: TGGAAAAAGGCCTAGACGGAG
DCD real-time probe: FAM-ACAGGCACCAAAGCCAAGGAAGCA-TAMRA

To control against amplification of genomic DNA, primers were designed to span exon boundaries. The 18S ribosomal RNA (rRNA) gene was used as an endogenous control to normalise for differences in the amount of total RNA in each sample. 18S rRNA primers and probe were purchased from Applied Biosystems. PCR reactions were performed using the ABI Prism 7500 fast Sequence Detection System (Applied Biosystems, Warrington, UK), according to the manufacturer's

[8]I acknowledge Dr. Caroline Pennington and Prof. Dylan Edwards, School of Biological Sciences, University of East Anglia, Norwich, UK, who perfomed these analyses.

gaccctagatcccaagatctccaaggatttggtggcatacccactccagcacacagaagcATGaggt
tcatgactctcctcttcctgacagctctggcaggagccctggtctgtgcct↑**atgatccagaggccgcct**
ctgccccaggatcggggaacc↑cttgccatgaagcatcagcagctcaaaaggaaaatgcagg
tgaagacccagggttagccagacaggcaccaaagccaaggaagcagagatccagccttctgg↑aa
aaaggcctagacggagcaaaaaaagctgtgggggactcggaaaactaggaaaagatgcagtcga
agatctagaaagcgtgggtaaagg↑agccgtccatgacgttaaagacgtccttgactcagtactaTAG
ctgtaaggagaagctgagaaatgatacccaggagcagcaggctttacgttttcagcctaaaacct

Figure 2.2 ***DCD* gene nucleotide sequence with marked targets for the real-time PCR primers and probe.**
↑ denotes site of an intron. DCD real-time PCR primer target singly underlined and probe target doubly underlined. PIF-CP nucleotide sequence in bold. Start and stop codons are shown in upper case.

instructions. Each reaction was performed in 25μL and contained the equivalent of 5ng of reverse transcribed RNA (1ng RNA for the 18S analyses), 50% TaqMan 2X PCR Master Mix (Applied Biosystems), 200nM each of the forward and reverse primer, and 100nM of probe. Conditions for the PCR reaction were: 2min at 50°C, 10 min at 95°C and then 40 cycles, each consisting of 15s at 95°C and 1 min at 60°C. The ABI Prism 7500 measured the cycle-cycle changes in fluorescence in each sample and generated a kinetic profile of DNA amplification over the 40-cycle PCR reaction. The cycle number (termed cycle threshold, or C_T) at which amplification entered the exponential phase was determined and this number was used as an indicator of the amount of target RNA in each tissue; that is, a lower C_T indicated a higher quantity of starting RNA. Relative and/or absolute standard curves for C_T versus input RNA were prepared, and relative and/or absolute levels of starting RNA in each sample were determined. Only DNA samples with an 18S C_T value within 1.5 C_Ts of the median C_T for 18S were used for analysis as this value suggested that the RNA was of sufficient quality for analysis of DCD expression. The C_T value of DCD was used to classify its expression as: very high (C_T <25.5), high (≥25.5 C_T <30.5), moderate (≥30.5 C_T <35.5), low/absent (≥35.5 C_T <40), or not detected/below the limits of detection (C_T = 40) [522]. Because of a drop off in sensitivity of the instrument, C_T values >35.5 are unreliable in terms of exact levels of mRNA expression or assessing changes in C_T values. The PC-3M cells transfected with the pcDNA3.1+DCD plasmid gave a C_T value of 23.1. Thus, the positive control was in the very high range of DCD mRNA expression. All *in vitro* experiments were performed in sextuplet and repeated three times.

2.7.4 Nucleotide sequencing

Nucleotide sequencing of PCR products was performed as a commercial service (MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK) using the *PIF* and *HCAP* primers.

2.8 Western blotting and co-immunoprecipitation

2.8.1 DGC study (Chapter 7)[9]

For immunoprecipitation reactions, muscles were homogenised in 1mL of immunoprecipitation lysis buffer (1% Triton X-100, 150mM NaCl, 50mM Tris-HCl [pH 7.5], 1mM ethylenediaminetetraacetic acid [EDTA], 1mM phenylmethanesulphonylfluoride [PMSF], with standard protease and phosphatase inhibitors), and 500μg of protein was used for each reaction. Extracts were precleared of non-specific immunoprecipitates with 1μg IgG for 1h at 4°C. Supernatants were transferred to a fresh tube, and 1μg of antibody was added to the tube and incubated by rocking overnight at 4°C. After immunoprecipitation, complexes were collected, washed, and resuspended in 30μL in standard loading buffer. Western blots were performed to probe for myofibrillar proteins as previously described [46], with the exception that α-dystroglycan (α-DG) incubations were done with 150mM NaCl, 20mM Tris (pH 7.4). Antibodies used were dystrophin (dys-2, carboxy specific; 1:50), ß-DG (1:100), α-sarcoglycan (α-SG) (1:200), ß-SG (1:200), and dysferlin (1:100) (all from Novocastra, Newcastle Upon Tyne, UK), MyHC I and II, and skeletal actin (all from Sigma-Aldrich, St.

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[9]I acknowledge Dr. Swarnali Acharyya and Prof. Denis Guttridge, Division of Cancer Genetics, The Ohio State University, Columbus, OH, USA, who performed these analyses.

Louis, MO, USA), and the 18.4 dystrophin rabbit pAb (carboxy specific; 1:50,000) (Cox et al., 1994). For enzymatic deglycosylation and dephosphorylation analysis, 20μg of tissue lysate was treated with either N-glycosidase F (PNGase F) or γ-phosphatase (New England Biolabs, Ipswich, MA, USA) respectively, according to the manufacturers' instructions.

2.8.2 PKR/ eIF2α study (Chapter 8)[10]

Muscle samples (approximately 10mg) were homogenised in 500ml of PhosphoSafe™ Extraction Reagent (Merck Biosciences, Nottingham, UK), and centrifuged at 15,000g for 15min. Samples of cytosolic protein (10mg) were resolved using 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (6% for eIF2α), and transferred to 0.45mm Hybond A nitrocellulose membranes (Amersham Biosciences Ltd, Bucks, UK), which had been blocked with 5% Marvel in Tris-buffered saline (pH 7.5) at 4°C for 1-2h. Membranes were then washed for 15min in 0.5% Tween-buffered saline or Tris-buffered saline (TBS)-Tween. Antibodies were rabbit pAbs to phospho PKR (pThr 446; 1:1000) (Insight Biotechnology, Wembley, UK) and total PKR (carboxy specific; 1:1000) (New England Biolabs, Herts, UK), rabbit polyclonal antisera to total eIF2α (1:1000) (Santa Cruz Biotechnology, CA, USA), rabbit polyclonal antisera to phospho eIF2α (1:1000) (Abcam, Cambridge, UK), mouse mAb to MyHC II (1:100) (Novocastra, Newcastle, UK), rabbit polyclonal antisera to actin (1:250) (Sigma Aldridge, Dorset, UK), peroxidase-conjugated goat anti-rabbit Ab (1:1000)

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[10]I acknowledge Dr. Helen Eley, Dr. Steven Russell and Prof. Michael, Tisdale, School of Life and Health Sciences, Aston University, UK, who performed these analyses.

and peroxidase-conjugated rabbit anti-mouse Ab (1:1000) (Dako Ltd, Cambridge, UK). Incubation with primary antibodies was at 4°C overnight, except for total eIF2α (1-2h at room temperature). Primary antibodies were washed off the membranes for 15min (changing the wash every 5min), except for actin, which was washed for 45min (changing the wash every 15 min). TBS-Tween (0.1%) was used for washing phospho antibodies and total antibodies. Secondary antibodies were used at a dilution of 1:1000, and were washed off after 45min. Development was by enhanced chemiluminescence (ECL) (development kits from Amersham Biosciences Ltd, Bucks, UK), and films were developed for 3-6min. Densitometry was used to quantify differences in protein levels.

2.9 Mass spectrometry of urinary proteins

2.9.1 1D SDS-PAGE gel electrophoresis

Preliminary work with 2D gels of urinary proteins demonstrated a low level of complexity compared to previous experience with plasma, serum and saliva; thus, 1D SDS-PAGE was used for initial separation (NuPAGE Novex 4-12% Bis-Tris gradient gels, Invitrogen, Paisley, UK). In order to avoid potential 'masking' of normal urinary proteins by novel species that may be highly prevalent in cancer patient urine, equal volumes (rather than equal amounts) of precipitated protein supernatant were loaded onto gels. Thirteen μL of protein sample were prepared with 5μL of 4X SDS sample buffer and 2μL of 10X reducing agent. Gels were loaded with low and high molecular weight markers (GE Healthcare UK Ltd, Little Chalfont, UK) and stained with GelCode Blue (Pierce, Loughborough, UK). Using

a clean scalpel, bands were excised in an identical fashion from each gel lane in seven separate blocks (see Figure 9.1, p.326).

2.9.2 In-gel tryptic digestion

Gel blocks were incubated 3 times in 200mM NH_4HCO_3 in 50% acetonitrile (ACN) at 30°C for 30min. Each block was incubated in 20mM DTT/200mM NH_4HCO_3 in 50% ACN at 30°C for 1h to reduce the protein, followed by three washes in 200mM NH_4HCO_3 in 50% ACN. Cysteines were alkylated by incubation in 50mM iodoacetamide/200mM NH_4HCO_3 in 50% ACN at room temperature for 20min in the dark, followed by 3 further washes. Gel blocks were cut into pieces and incubated with 100% ACN until white. Gel pieces were then air-dried before incubation with 0.5µg trypsin (Promega, Southampton, UK) in 50mM NH_4HCO_3 for 16h.

2.9.3 Matrix-assisted laser desorption/ionisation mass spectrometry

Tryptic digests were sonicated for 10min. Samples were passed through zip-tip pipette tips (C18 Zip-Tip, Millipore, Watford, UK) to reduce salt contamination. Aliquots of the tryptic digests (0.5µL) were mixed with 0.5µL of 10mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) matrix, and analysed using a Voyager DE-STR matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometer (MS) (Applied Biosystems, Warrington, UK). Processed spectra were searched against the Swissprot and National Center for Biotechnology Information (NCBI) non-redundant databases using Protein Prospector MSFit and Mascot PMF (Matrix Science, London, UK). Search parameters included a maximum of 1

missed cleavage per peptide and a mass tolerance of 100 parts per million (ppm). Proteins with a significant Molecular Weight Search (MOWSE) score ($p<0.05$) are reported.

2.9.4 Liquid chromatography tandem mass spectrometry

Tryptic digests were sonicated for 10min. Samples were passed through zip-tip pipette tips (C18 Zip-Tip, Millipore, Watford, UK) to reduce salt contamination. For liquid chromatography tandem MS (LC-MS/MS), tryptic digests from gel blocks were pooled into two groups by molecular weight (MW): MW ≤30kD and MW >30kD. Pooled samples were spun through a 0.22μm filter (Millipore, Watford, UK). One hundred μL of each pooled sample were loaded onto a PepMap C18 column (3μm particle size, 75μm x 15cm) on a Famos/Swichos/Ultimate high performance liquid chromatography (HPLC) system (Dionex, Camberley, Surrey, UK) at a flow rate of 200nL/min. The column was equilibrated with solvent A (0.1% formic acid) and eluted with a linear gradient from 0 to 70% solvent B (0.1% formic acid in 100% ACN) over 45min. Flow was controlled by Chromeleon software and interfaced to the LCQ deca MS (Thermo Electron, Auchtermuchty, Fife, UK) with a PicoTip (FS-360-20-10-N-20-C12 DOM, New Objective, Basingstoke, UK). Output was monitored at 214nm. Data dependent acquisition was controlled by Xcalibur software, and database searching was achieved using TurboSequest software and Mascot. Redundancy was avoided within the protein counts by identifying the gene of origin of hypothetical and KIAA proteins wherever possible.

2.10 Assessment of physical activity

2.10.1 Accelerometer-based activity monitoring

Objective assessment of PA was performed using the activPAL™ PA meter (dimensions: 35x53x7mm; mass: 20g; PAL Technologies Ltd, Glasgow, UK), which uses a uni-axial accelerometer sampling at 10 hertz (Hz) to produce signals reflecting thigh inclination and movement with offline algorithms to generate an activity record on a second-by-second basis. A Universal Serial Bus (USB) interface docking station connects the monitor to a Windows-based computer and software package that classifies positions and activities into three categories: lying or sitting, standing and stepping (Figure 2.3). Acceleration signals exceeding particular peak acceleration amplitudes are registered as steps. Cadence and number of steps taken describe the intensity and volume of activity. The software assigns each activity an estimated energy cost in metabolic equivalents (METs) [524], representing the ratio of the active to resting metabolic rate, which are then summated over the assessment period to derive a value in MET.hrs that reflects overall free-living EE. One MET is equivalent to 1kcal/kg of body weight/hour (BMR). Lying or sitting is assigned an energy cost of 1.25METs, quiet standing 1.4METs and walking at 120steps/min 4METs. EE of stepping is scaled linearly according to the equation:

EE (in MET.hrs) = (1.4d) + (4-1.4) x (c/120) x d

where c = cadence (steps/min)

d = activity duration (hrs)

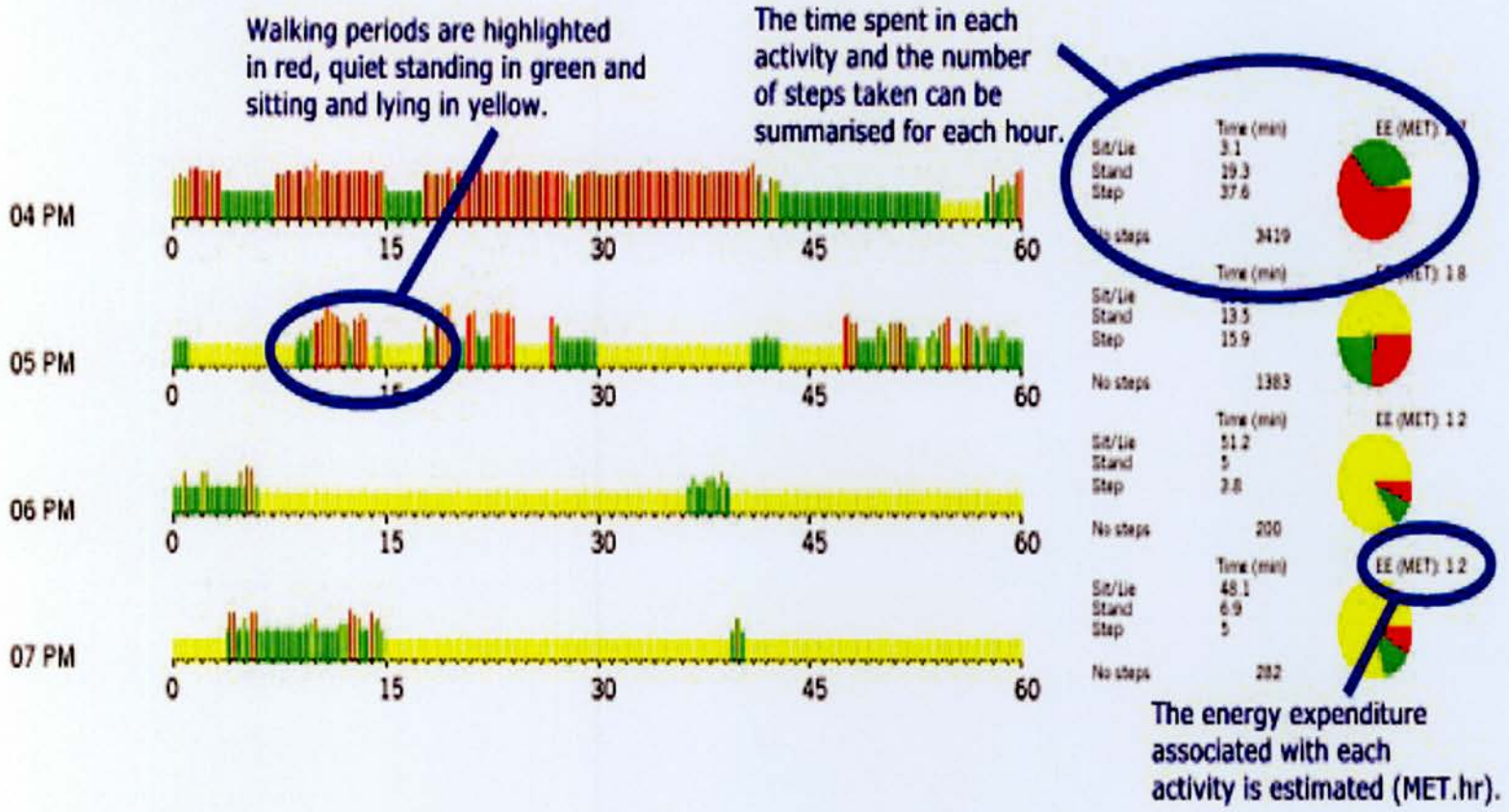

Figure 2.3 **Computer readout of data recorded by activPAL™.**
Taken from the PAL Technologies website (http://www.paltech.plus.com).

By recording the duration and intensity of activity categories, the activPAL™ system summates estimated EE over the assessment period. Data extracted were averaged over the length of a recording period to obtain average values per day. In the case of step cadence, data were analysed by comparing median step cadence during a recording period. To derive energy expenditure of activity (EEA), in the validation study of Chapter 10 ($EEA_{ActivPAL}$), the number of METs awarded to represent REE (i.e. 1.0 x 24 = 24METs/day) was subtracted from the total number of METs/day, This definition of EEA includes dietary-induced thermogenesis and non-exercise activity thermogenesis [525]. In Chapter 11 (in which 'EEA' is simply used as a measure of global PA), the number of METs recorded for non-activity (i.e. 1.25 x 24 = 30METs/day) was subtracted from the total number of METs/day. In all studies, an activPAL™ was attached to the subject's anterior mid-thigh with adhesive dressings (Figure 2.4). Subjects were allowed to remove activPAL™ during water-related activities.

2.10.2 Indirect calorimetry assessment of resting energy expenditure

Following an overnight fast, subjects attended at 0800. Subjects rested in a supine position for at least 30min before undergoing indirect calorimetry for 30min using a ventilated hood technique (GEM; NutrEn Technology Ltd, Lancs, UK). This system provides measurements of VO_2 and VCO_2 (L/min) with errors of less than 2.3% [526]. Measurements performed in the last 20 min were averaged to calculate REE using the Weir equation [527]:

$$REE = [VO_2 (3.94) + VCO_2 (1.11)] \times 1440$$

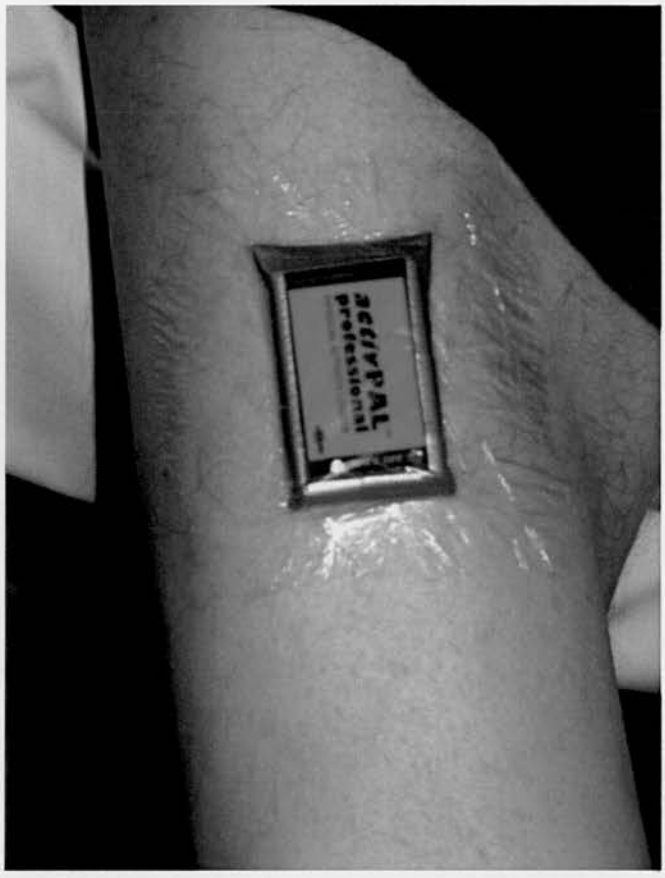

Figure 2.4 **ActivPAL™ meter attached to a patient's anterior thigh.**
The meter was attached in a sandwich of waterproof dressings. Firstly, a dressing was applied to the thigh to protect the skin. A further dressing was then applied over the meter to affix it in place. The patient was instructed how to remove and re-attach the meter, so that they could remove it for bathing, if desired. However, they were also instructed to re-attach the meter as soon as the thigh had been towelled dry.

2.10.3 Doubly labelled water assessment of total energy expenditure

2.10.3.1 Preparation of doubly labelled water

^{2}H-H_2O and ^{18}O-H_2O doses were made from a common stock for the study which was optimised for a body weight of 70kg and assuming 40kg TBW[11]. Doses were prepared from 10% ^{18}O-H_2O and 100% ^{2}H-H_2O to give an initial enrichment of 125ppm excess in body water. Each dose was aliquotted into 125mL leakproof wide neck polypropylene bottles (#2105-0004, Nalgene, Rochester, NY, USA) and weighed to 4 decimal places (dp). All were within 1% of a target weight of 48g. Doses were stored at -20^{O}C until required. No weight loss on freezer storage was observed. The final aliquot (or incomplete dose) was used to prepare a 500-fold gravimetric dilution (0.1g in 50g, weighed to 4dp) with tap water. Aliquots of this 'diluted dose' and the local tap water were retained for analysis.

2.10.3.2 Doubly labelled water protocol

On day 0, subjects collected an aliquot of the second urine sample of the day in a 30mL universal container and recorded the time. The DLW was then consumed and the bottle rinsed with tap water and the contents re-drunk to ensure that all DLW had been ingested. The time, date and unique code of the ingested dose were recorded. On days 1, 2, 3, 7, 12, 13 and 14, aliquots of the second urine samples of the day were collected and frozen at -20°C prior to analysis.

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[11]I acknowledge Ms. Alexandra Small and Prof. Tom Preston, Scottish Universities Environmental Research Center, The University of Glasgow, East Kilbride, UK, who prepared the DLW doses and perfomed the MS analysis of the DLW urine samples.

2.10.3.3 Deuterium and oxygen-18 analysis of urine samples

^{2}H analysis was performed according to the method of Scrimgeour *et al* [528], as described in Moses *et al* [277], with certain improvements[11]. Each sample tube was automatically overgassed with a 100mL/min flow of 20% hydrogen in helium for 40s (Air Products Special Gases, Crewe, UK) on a 220-place manifold. ^{2}H abundance was measured by continuous flow isotope ratio MS (CF-IRMS) with reference to working water standards, which had been calibrated against international standards. To ensure temperature stability, tubes were equilibrated beside this instrument within an air-conditioned instrument laboratory. After ^{2}H analysis, ^{18}O analysis was performed according to the method of Prosser *et al* [529]. Samples were overgassed with a 100mL/min flow of 3% CO_2 in nitrogen for 40s (Air Products Special Gases, Crewe) on the 220-place manifold. Reference samples (0 and 150ppm excess ^{18}O) were prepared and analysed with each batch. Samples were left to equilibrate for 24h at ambient temperature. The abundance of ^{18}O in CO_2 was measured by CF-IRMS and calculated according to the known abundance of reference waters. For both ^{2}H and ^{18}O analyses, additional water samples were included in each sample batch for quality control purposes. ^{2}H and ^{18}O adundances of the independent quality control samples were typically within 1ppm and 0.5ppm, respectively, of the accepted values.

2.10.3.4 Calculation of total energy expenditure

'Multipoint' calculations were used to derive turnover rates and initial enrichments of each isotope, to estimate CO_2 production and TBW, respectively[11]. Schoeller's equation for estimating TEE was used in the form given by Goran *et al* [530]. A

resampling procedure was used to estimate errors in TBW and TEE measurement [531]. The precision of TBW analysis was 0.11kg (standard deviation [SD]). TEE errors estimated by the resampling procedure averaged 3.1% (76.43kcal/d [SD 28.66]). Tracer elimination rate was normal (k_O/k_H =1.289, SD 0.051) and the average $^{2}H:^{18}O$ distribution volume or pool space ratio was 1.0294 (SD 0.0123). Predicted values for TEE were derived from predicted REE values [532] multiplied by 1.5. This prediction derives from the lifestyle category defined as 'Seated work with no option of moving around and little or no strenuous activity' given a PAL range of 1.4–1.5 by Black *et al* [512]. LBM was calculated from TBW data by assuming a hydration factor of 0.732 [533]. Body fat was calculated by difference (body weight - LBM). EEA was calculated from the formula:

EEA = TEE – REE

This definition of EEA also includes dietary-induced thermogenesis and non-exercise activity thermogenesis [525]. PAL was calculated from the formula:

PAL = TEE/REE

2.11 Questionnaires

2.11.1 Quality of life and physical functioning

During each activPAL™ recording period, subjects completed health-related QoL and mood questionnaires (see Appendix III, p.485). The EORTC QLQ-C30 (version 3.0) questionnaire was used to assess functional, symptomatic and global

QoL scores [534-536]. The FAACT (version 4) and FACIT-Fatigue (FACIT-F) (version 4) questionnaires were used to report anorexia/cachexia and fatigue scores using the appropriate 'Trial Outcome Index' (TOI), which summates the scores for physical well-being, functional well-being and the domain of interest (i.e. anorexia/cachexia or fatigue) [537].

2.11.2 Mood

During each activPAL™ recording period, mood was assessed using the Hospital Anxiety and Depression Scale (HADS) questionnaire (see Appendix III, p.485), which provides symptom scores for both anxiety (HADS-A) and depression (HADS-D) [538].

2.12 Statistical analysis

All statistical analyses were performed using Statistical Package for Social Services (SPSS) version 13.0 or version 15.0 (Chicago, IL, USA). Specific details on methods of data analysis for each study are found in the Results chapters. Statistical significance was set at the $p<0.05$ level for all tests. Quoted p-values are two-tailed.

Part B – Mediators of Muscle Wasting in Cancer Cachexia

Chapter 3 – Tumour-Derived Mediators I: Variation in dermcidin expression (as a surrogate for PIF expression) in a range of primary human tumours and human cell lines

3.1 Introduction

Cachexia is considered the result of an interaction between tumour-derived and host-derived mediators (Chapter 1.4). Part B focuses on the investigation of tumour-derived mediators of muscle wasting, including PIF and MIC-1, and host-derived mediators, including sex steroids. In Chapters 3 and 4, the expression of DCD mRNA, as a proxy of PIF expression, is studied in human cell lines and tumour samples.

DCD and its protein products have been investigated from a number of different scientific angles and, consequently, they have received several different but synonymous names. The *DCD* gene was identified on chromosome 12q13.1 and encodes different proteins with divergent biological functions (see Figure 1.6, p.72) [194]. The 110-amino acid DCD polypeptide [194] is identical to HCAP [197] and DSEP [201]. The protein products of the DCD peptide include the 47-amino acid DCD-1 peptide (a skin antimicrobial) [194] and the 30-amino acid PIF-CP [206], the latter of which is identical to Y-P30 [200]. When glycosylated, PIF-CP is reported to behave as a pro-cachectic factor in cancer [17], capable of inducing skeletal muscle proteolysis in animal models [539] and cultured myotubes [540]. However, control of expression and post-translational processing of DCD into its

various subunits (including PIF) has not yet been fully elucidated (see Figure 1.6, p.72).

DCD acts as a survival factor in prostate cancer cells exposed to either hypoxia or oxidative stress [541], and hepatoma cell lines subject to oxidative stress [206]. Porter *et al* determined that DCD expression in breast cancer cell lines was associated with cancer cell growth and survival [195]. As such, *DCD* has been suggested as a candidate oncogene in breast cancer [195]. Cunningham *et al* studied the role of oxidative stress in neuronal degeneration and identified a 30 amino acid survival-promoting peptide, which they named Y-P30 [200]. The Y-P30 peptide was present in medium conditioned by human retinoblastoma cells and a mouse hippocampal cell line exposed to hydrogen peroxide. Y-P30 was subsequently identified as comprising part of the 110-amino acid DSEP polypeptide (identical to DCD) [201]. DSEP-overexpressing neuronal cells were also found to have an increased resistance to oxidative stress [201]. Thus, Y-P30 and PIF-CP represent different names (and functions) for the same peptide. When non-glycosylated, the peptide appears to behave as a tumour survival factor (Y-P30) [200], and when glycosylated, it has been proposed as a pro-cachectic factor (PIF) [17]. However, the existence of a functional human PIF has been called into question recently. The previous studies focused on cancer cachexia have all employed a mAb that may not be specific to PIF, and might also bind albumin and immunoglobulins [208]. Furthermore, using this mAb, PIF immunoreactivity was not associated specifically with cancer, skeletal muscle wasting or survival [208]. Thus, there are no validated or specific methods with which to prove conclusively the existence of a human PIF.

As DCD appears to encode for both putative cachectic and tumour survival factors (see Figure 1.6, p.72), it represents an important potential therapeutic target in cancer patients. The expression of DCD mRNA has been used previously as a proxy for PIF expression in studies of prostate cancer (DCD mRNA was detected in most prostate cancer cell lines, primary prostate tumours and bony metastases) [197]. Thus, the present study aimed to evaluate the expression of DCD mRNA (as a surrogate of PIF-CP expression) using quantitative real-time PCR in a range of malignant human tumours (specifically OGC, pancreatic, bile duct and prostate cancer) and human cell lines (pancreatic cancer and prostate cancer cell lines). Upper GI cancer types are associated significantly with the development of cancer cachexia [4], whereas prostate cancer represents the first tumour type in which DCD expression was analysed (although the prevalence of cachexia in prostate cancer is lower than that seen in upper GI cancer) [197].

3.2 Hypothesis

DCD mRNA is expressed at high levels in tissue samples from malignant human tumours (including oesophageal, gastric, pancreatic/bile duct and prostate tumours) and human cell lines (including pancreatic cell lines and some prostate cell lines), but is not expressed by benign human tissue.

3.3 Patients, healthy controls and cell lines

Upper GI cancer patients and healthy controls were recruited (see Methods Chapter 2.1, p.167). Tissue samples were obtained from patients with oesophageal cancer (n=28); gastric cancer (n=20); benign oesophago-gastric tissue (n=10); a range of benign and malignant pancreatic/bile duct masses (n=5 pancreatic adenocarcinoma, n=2 chronic pancreatitis, n=1 cholangiocarcinoma, n=1 liposarcoma, n=1 adenoma, and n=1 cystadenoma); prostate cancer (n=8 Gleason six, n=31 Gleason seven, n=3 Gleason eight, n=6 Gleason nine, and n=4 Gleason ten); and BPH (n=16) (see Methods Chapter 2.4.2 and 2.4.3, p.174). Pancreatic (MIA-Pa-Ca-2 and CFPAC) and prostate cancer cell lines (hormone-sensitive PC-3M and LNCaP; hormone-insensitive PC-3 and DU145) were also included (see Methods Chapter 2.6.1, p.176). Quantitative DCD mRNA expression was analysed in tissue samples and cell lines using real-time PCR (see Methods Chapter 2.7.3, p.182). The C_T value of DCD was used to classify its expression as: very high (C_T <25.5), high (≥25.5 C_T <30.5), moderate (≥30.5 C_T <35.5), low/absent (≥35.5 C_T <40), or not detected/below the limits of detection (C_T = 40) [522]. PC-3M cells transfected with the pcDNA3.1+DCD plasmid were used as a positive control (see Methods Chapter 2.6.2, p.178), and gave a C_T value of 23.1. Thus, the positive control was in the very high range of DCD mRNA expression. All *in vitro* experiments were performed in sextuplet and repeated three times.

3.4 Statistical analysis

Where appropriate, results were expressed as means.

3.5 Results

3.5.1 DCD expression levels in oesophageal and gastric tissue samples

Table 3.1 details the DCD expression levels of all of the clinical tissue samples. Nine (19%) of the 48 OGC samples displayed detectable levels of DCD expression (C_T<40), including 4/28 (14%) oesophageal and 5/20 (25%) gastric tumours. However, only 2/48 (4%) of the tumour samples produced a DCD C_T value <35.5, representing reliable moderate levels of DCD expression (2 gastric adenocarcinomas with C_T values of 30.5 and 31.7, respectively). None of the 10 benign control samples demonstrated detectable levels of DCD mRNA.

3.5.2 DCD expression levels in pancreatic cell lines and tissue samples

3.5.2.1 DCD mRNA expression in pancreatic cell lines

Both pancreatic cancer cell lines used in this study demonstrated DCD mRNA expression. MIA-Pa-Ca-2 and CFPAC cell lines had C_T values of 35.1 and 37.6 (i.e. moderate and low/absent levels of DCD mRNA expression), respectively.

3.5.2.2 DCD mRNA expression in pancreatic tissue samples

Of the pancreatic/bile duct tissue samples, 5/11 (45.5%) expressed DCD mRNA, 3 of which had C_T values 25.5-30.5, defined as high levels of expression (2 metastatic pancreatic adenocarcinomas and 1 poorly differentiated cholangiocarcinoma). Overall, 3/5 (60%) pancreatic adenocarcinoma samples had moderate or high levels of DCD expression. The histopathology and C_T values of pancreatic/bile duct tissue

Organ	Number of samples			Dermcidin expression level					
				Sample type	Number of samples				
	Benign	Cancer	Cell lines		Very high n (%)	High n (%)	Moderate n (%)	Low/ absent n (%)	Not detected/ below limits of detection n (%)
Oesophagus	5*	28	0	Benign:	0	0	0	0	5 (100)
				Cancer:	0	0	0	4 (14.3)	24 (85.7)
Stomach	5*	20	0	Benign:	0	0	0	0	5 (100)
				Cancer:	0	0	2 (10)	3 (15)	15 (75)
Pancreas	2†	5	2	Benign:	0	0	0	0	2 (100)
				Cancer:	0	2 (40)	1 (20)	0	2 (40)
				Cell Line:	0	0	1 (50)	1 (50)	0
Bile duct	0	1	0	Cancer:	0	1 (100)	0	0	0
Prostate	16‡	52	4	Benign:	0	0	0	0	16 (100)
				Cancer:	0	0	0	0	52 (100)
				Cell Line:	0	0	0	3 (75)	1 (25)
Others	2**	1††	0	Benign:	0	0	0	0	2 (100)
				Cancer:	0	0	0	1 (100)	0

Table 3.1 **Levels of dermcidin mRNA expression in primary tissue and cell lines for each organ site.**
Only pancreatic and bile duct cancer samples demonstrated relatively high frequencies of either moderate or high dermcidin mRNA expression. *Benign samples taken during normal upper GI endoscopy examinations; †chronic pancreatitis samples; ‡BPH samples; **pancreatic cystadenoma and adenoma; ††pancreatic liposarcoma.

samples that expressed DCD mRNA are shown in Table 3.2.

3.5.3 DCD expression levels in prostate cancer cell lines and tissue samples

3.5.3.1 DCD mRNA expression in prostate cancer cell lines

The prostate cancer cell lines that were investigated demonstrated only low or absent levels of DCD expression. PC-3 had a C_T value of 36.7, LNCaP a C_T of 39.2 and DU145 a C_T of 39.6. PC-3M had a C_T value of 40.

3.5.3.2 DCD mRNA expression in prostate cancer tissue samples

None of the 68 prostate tissue samples, either benign or malignant, expressed DCD mRNA (C_T values of 40).

Pathology	Disease stage	DCD C_T value	Level of expression
Gall bladder metastasis from pancreatic ductal adenocarcinoma	IV	26.7	High
Metastatic pancreatic adenocarcinoma (known liver metastasis)	IV	29.3	High
Cholangiocarcinoma (poorly differentiated adenocarcinoma with perineural and microvascular/lymphatic invasion)	IVa	29.7	High
Pancreatic adenocarcinoma (moderately differentiated)	IIa	30.9	Moderate
Liposarcoma	N/A	35.6	Low/Absent

Table 3.2 **Histopathological and staging details of the patients from which hepatopancreaticobiliary tissue samples were analysed for expression of dermcidin mRNA.**
The samples listed are those that appeared to express DCD mRNA.

3.6 Discussion

The present study demonstrated a wide range of levels of DCD mRNA expression across different cancer types, namely prostate, gastric, oesophageal, pancreatic and bile duct cancer. DCD mRNA expression was undetectable in the primary prostate cancer samples. In contrast, 60% of pancreatic cancer samples expressed significant quantities of DCD mRNA. Additionally, the MIA-Pa-Ca-2 pancreatic cancer cell line was the only cell line that reliably expressed borderline moderate levels of DCD mRNA.

Assessment of 58 gastric and oesophageal biopsy samples showed that DCD mRNA expression occurred in a small percentage of OGC and that expression levels were minimal. (Only 2 (4%) OGC samples expressed moderate levels of DCD mRNA). Thirty-three of the 48 (68.8%) gastric and oesophageal cancer patients were weight-losing (median weight loss of the whole group = 3.5%; range 0.0-23.2), and are described in detail later in this thesis. However, the 9 OGC patients with tumours that demonstrated any level of DCD expression did not have significantly greater weight loss, lower BMI or higher CRP than those patients not expressing DCD (data not shown). However, with such low numbers of DCD-expressing tumours, it is difficult to make robust conclusions regarding differences in patient characteristics between groups.

Some of the OGC samples utilised in this study have been included in previous work published from the author's laboratory [542]. Deans *et al* found a higher percentage of PIF-CP mRNA-expressing OGC samples than in the present work.

The previous analysis used the MIA-Pa-Ca-2 cell line as a positive control from which to compare relative expression of DCD mRNA [542]. In the present study, MIA-Pa-Ca-2 was found to have a C_T value of 35.1, equating to borderline moderate DCD expression. Due to the limited expression of this cell line, MIA-Pa-Ca-2 was not chosen as a positive control in the present study. Thus, PIF-CP expression may have been overestimated in the previous study due to comparison with a control that has only a limited level of DCD mRNA expression. In the current study, mRNA from PC-3M cells stably transfected with DCD was used to confirm DCD mRNA expression for real time PCR. However, as DCD had been artificially overexpressed in the PC-3M cell line, this was not an acceptable positive control for use in a relative gene expression method of real-time data expression, as it does not bear any relationship to the *in vivo* situation. Thus, C_T values and DCD/18S rRNA ratios were used in this study to assess DCD mRNA expression.

Results from prostate tissue samples suggest that DCD was not expressed by primary prostate cancer. Moreover, the immortalised prostate cell lines that did express DCD, only did so at a very low/absent level. Other sources have suggested previously that DCD is expressed by prostate cancer. In agreement with the data presented above, Wang *et al* demonstrated DCD/HCAP mRNA expression in prostate cancer cell lines other than PC-3M [197]. However, Wang and co-workers also identified DCD/HCAP mRNA in primary prostate cancer tumours and secondary bone metastases, results that differ from those presented here. One possible explanation for these differences is that Wang *et al* used RT-PCR rather than real-time PCR, and thus Wang was only able to qualify DCD mRNA

expression but was unable to quantify this as being of only negligible amount. Another possible explanation is that, although the present study included a large number of patient samples from five tumour types, plus benign tissue from some of the affected organs, there were no samples taken from distant metastatic deposits, which have been shown to express high levels of DCD in previous studies [197].

A further hypothesis underlying the lack of DCD mRNA expression in the prostate cancer samples analysed in the present study is selective degradation of DCD mRNA. A major control step in gene expression is the turnover of mRNA, a tightly regulated process. Important *cis*-acting elements controlling the half-life of mRNA are adenylate- and uridylate-rich (AU-rich) elements (AREs) found in the 3' untranslated regions (UTRs) of many unstable mRNAs [543]. AREs usually contain repeats of AUUUA, and inclusion of AUUUA motifs within 3'UTRs of mRNA may accelerate their decay. Dermcidin has one AUUUA pentamer within its 3'UTR (http://www.genomatix.de), which may make DCD mRNA more susceptible to degradation. However, for destabilising proteins to form stable complexes with ARE-containing mRNAs, many AUUUA pentamers are usually required [543].

In this study, absolute C_T values were used to assess DCD mRNA expression. Due to a drop off in the sensitivity of the instrument, C_T values $\geq$35.5 are unreliable in terms of the amount of mRNA expressed. Furthermore, the comparison of C_T values $\geq$35.5 is not reliable, as differences in C_T are unlikely to represent real or meaningful differences in the amount of mRNA present. In primary tumour

samples, which have a large degree of cellular heterogeneity, C_T values $\geq$35.5 mRNA represent low expression. Such C_T values may reflect generally low expression or there may be a subset of cells expressing an appreciable level of the mRNA. However, in cell lines, where heterogeneity is not such an issue, a C_T value $\geq$35.5 means that mRNA may be absent or expressed at only a very low level on a per cell basis.

For the current study, the presence of DCD was assessed using mRNA rather than protein levels. A DCD mAb called G-81 is available. However, G-81 recognises the carboxy-terminus of DCD-1 rather than full length DCD or PIF-CP/Y-P30 [544]. Polyclonal antibodies to DCD also exist, but, in the past, it has not been possible to use these to identify successfully DCD or PIF-CP. PIF is also reported to exist in a glycosylated form and is a putative factor in the increased muscle proteolysis/decreased protein synthesis of cancer cachexia [545]. As the existing mAb directed against PIF is not specific to glycosylated PIF alone but may also recognise carbohydrate epitopes on other molecules [208], it was not used in the present study. The rationale for using DCD mRNA expression as a proxy of PIF expression is based on the facts that DCD/HCAP shares homology with murine PIF, and that such a technique has been utilised previously. Further supportive evidence for a role for DCD in cachexia comes from the observation that DCD has been shown to induce lipolysis in normal mice via TNF-α-induced downregulation of perilipin in adipose tissue [546]. However, at present, there is no conclusive evidence that human PIF-CP is indeed a downstream proteolytic product of DCD. If PIF-CP protein expression was to be confirmed in humans, it seems likely that

differential proteolysis of DCD would be the underlying regulatory mechanism, as the downstream sweat peptide products of DCD all appear to be proteolytic in origin [547]. However, the subsequent glycosylation of PIF-CP to create a human PIF with functional proteolytic action would still be in question.

In summary, the results presented here demonstrate that DCD mRNA expression varied between tumour types and within primary tumours of the same type. The substantial variability of DCD expression in primary tumours and cell lines demands development of accurate and novel detection methods for the DCD/PIF protein itself. The lack of such methods makes the evaluation of the role of DCD/PIF in human tumour biology elusive. However, it would appear, based on mRNA data that, the expression of DCD/PIF is very infrequent in OGC and prostate cancer, but may be more prevalent in pancreaticobiliary tumours. Thus, if PIF is a downstream product of DCD, PIF might account for some of the skeletal muscle wasting associated with the latter tumours. It remains possible that even low levels of DCD expression by a subset of cancer cells may be sufficient to promote clonal cancer cell survival in an adverse environment and the subsequent induction of a severe cachectic phenotype. As such, further studies of DCD expression in human pancreatic cancer and the relationship with patient phenotype and outcome may be the optimum way of taking the biology of human DCD forward, in the absence of an accurate methodology for PIF detection.

This chapter utilised real-time PCR as the investigative method of choice for DCD mRNA detection. In Chapter 4, RT-PCR is utilised instead, in order to generate

cDNA that can be sequence analysed to investigate the potential for PIF glycosylation.

Chapter 4 – Tumour-Derived Mediators II: Sequence analysis of the dermcidin gene for PIF glycosylation sites in human cancer cell lines

4.1 Introduction

Using the PIF mAb, urinary PIF immunoreactivity has been reported in 80% of patients with advanced pancreatic cancer [183] and in 56% of patients with advanced OGC [208]. However, PIF immunoreactivity was not specific to cancer patients and was not necessarily associated with clinical outcomes (e.g. weight loss, skeletal muscle wasting or survival) [208]. Furthermore, the PIF mAb has demonstrated non-specific binding activity with other proteins [208]. Thus, controversy surrounds the role of PIF in human cancer cachexia.

Purified murine PIF is resistant to trypsin, has a mass of ~24kDa, and consists of a short polypeptide core (3kDa), an *N*-linked sulphated oligosaccharide chain (~10 kDa), and an *O*-linked sulphated oligosaccharide chain (~6 kDa) [203]. However, the exact nature of the carbohydrate structures that confer the proteolysis-inducing activity to this unusual molecule remain unresolved [203]. The sites at which glycosylation occurs to form the functional glycoprotein have not been established. Furthermore, there are a number of outstanding questions related to the peptide core. A peptide sequence and two patents describing the HCAP gene encoding PIF and its putative peptide products have been published previously (Genebank accession number AR053250) [197]. Whilst the sequence of the PIF-CP was novel at the time, there have been several reports of other molecules encoded by the same gene (see

Figure 1.6, p.72) [194, 201]. The organisation of the dermcidin gene should allow discrete expression of molecules with various functions. However, the absence of the *N*-glycosylation site seen in murine PIF (amino acids 13-15, Asn-Pro-Ser) within the human sequence (Asn-Pro-Cys) has raised doubt as to whether the human homologue can be glycosylated. It is worth noting that a serine/cysteine substitution mutant of PIF protein transfected into and expressed in human immortalised cell lines was not glycosylated [204].

It has been hypothesised that the presence of polymorphic or mutant species of DCD/PIF mRNA may make glycosylation, and hence proteolytic activity, of the resultant PIF possible within human tumours. In order to assess the likelihood that the PIF-CP contained within DCD can be glycosylated in humans, the present study aimed to use RT-PCR, followed by nucleotide sequencing of the resultant PCR products, to investigate the presence or absence of DCD gene sequence variants in human cancer cell lines, including pancreatic (CFPAC, PANC-1 and MIA-Pa-Ca-2), hepatocellular (Hep G2 and Huh-7), prostatic (PC-3M, LNCaP, PC-3, DU145) and malignant melanoma (G361) cell lines. Upper GI cancer cell lines were chosen as these types of cancer are associated significantly with the development of cancer cachexia [4], whereas prostate cancer represents the first tumour type in which DCD mRNA expression was analysed [197]. The malignant melanoma line G361 was chosen for investigation as it has been reported to produce a glycosylated human homologue of PIF (based on PIF mAb immunoreactivity), which induces skeletal muscle wasting in nude mice [198].

4.2 Hypothesis

Human cancer cell lines express polymorphic or mutant species of DCD mRNA that make glycosylation of the resultant PIF-CP possible.

4.3 Cell lines

DCD mRNA expression was analysed in human cancer cell lines using RT-PCR (see Methods Chapter 2.7.2, p.179). Cell lines included were pancreatic (CFPAC, PANC-1 and MIA-Pa-Ca-2), hepatocellular (Hep G2 and Huh-7), prostatic (hormone-sensitive PC-3M and LNCaP; hormone-insensitive PC-3 and DU145), and malignant melanoma (G361) cell lines (see Methods Chapter 2.6.1, p.176). PC-3M cells transfected with the pcDNA3.1+DCD plasmid were used as a positive control (see Methods Chapter 2.6.2, p.178).

4.4 Statistical analysis

Not applicable (N/A).

4.5 Results

4.5.1 Expression of DCD mRNA by human cancer cell lines

Compared with the positive control created by stably transfecting PC-3M cells with a DCD-expressing vector, DCD mRNA expression was detected consistently in multiple human cancer cell lines by RT-PCR. All of the results reported are from cDNA specimens with confirmed expression of an appropriate housekeeping gene, and two different sets of PCR primers (*PIF* and *HCAP*) used to identify DCD mRNA. Subsequent PCR product nucleotide sequencing confirmed the identity of the amplified products.

The *PIF* primers amplified a band of the appropriate size (174bp) in 8 of the 10 human cancer cell lines assessed, including PC-3, PC-3M, DU145, G-361, Hep G2, CFPAC, PANC-1 and MIA-Pa-Ca-2 (Table 4.1 and Figure 4.1). A 174bp product was not detected in LNCaP and Huh-7 cell lines using the *PIF* primers. *HCAP* primers amplified a band of appropriate size (361bp) in 6 of the 10 cell lines, including PC-3, PC-3M, DU145, Hep G2, CFPAC, and MIA-Pa-Ca-2 (very weak band) (Table 4.1 and Figure 4.2). A 361bp product was not detected in G-361, PANC-1, LNCaP and Huh-7 cell lines using the *HCAP* primers. Thus, in G-361 and PANC-1 cell lines, an appropriately sized product could only be demonstrated by the *PIF* primers and could not be confirmed by the *HCAP* primers. This finding was consistent over 5 replications of the corresponding PCR reactions.

	Amplification of DCD nucleotide sequence			
Cell line	***PIF* primers**		***HCAP* primers**	
	PCR band	**DCD sequence**	**PCR band**	**DCD sequence**
DCD positive vector	✓	✓	✓	✓
LNCaP	x	x	x	x
PC-3	✓	✓	✓	✓
PC-3M	✓	✓	✓	x
DU145	✓	✓	✓	x
G-361	✓	✓	x	x
Hep G2	✓	✓	✓	x
Huh-7	x	x	x	x
CFPAC	✓	✓	✓	x
PANC-1	✓ (weak band)	x	✓ (weak band)	x
MIA-Pa-Ca-2	✓	✓	✓ (weak band)	x

Table 4.1 **Results of RT-PCR of immortalised human cell lines using *PIF* and *HCAP* primers.**
Three of the cell lines have been reported to be cachexia-inducing in immunocompromised mice, namely G-361 (34), PC-3M (25), and DU145 (37). Only LNCaP and Huh-7 cell lines demonstrated no evidence of DCD mRNA expression with both *PIF* and *HCAP* primers.

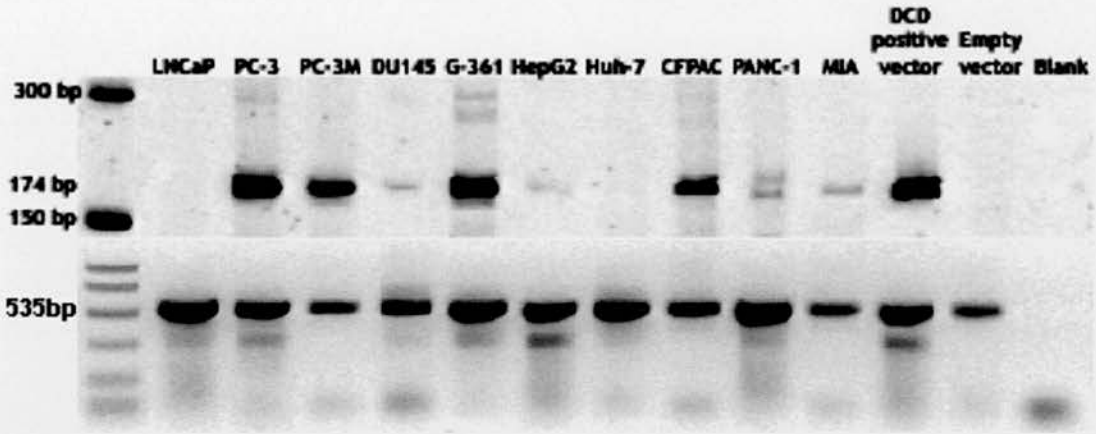

Figure 4.1 **RT-PCR of immortalised human cancer cell lines using *PIF* (upper gel) and *β actin* (lower gel) primers.**
DCD cDNA product at 174bp. Bands were demonstrated for PC-3, PC-3M, DU145, G-361, Hep G2, CFPAC, PANC-1 and MIA-Pa-Ca-2 at the same level as DCD positive control. All products were identified as DCD cDNA on nucleotide sequencing. β actin cDNA product at 535bp present for all cell lines.

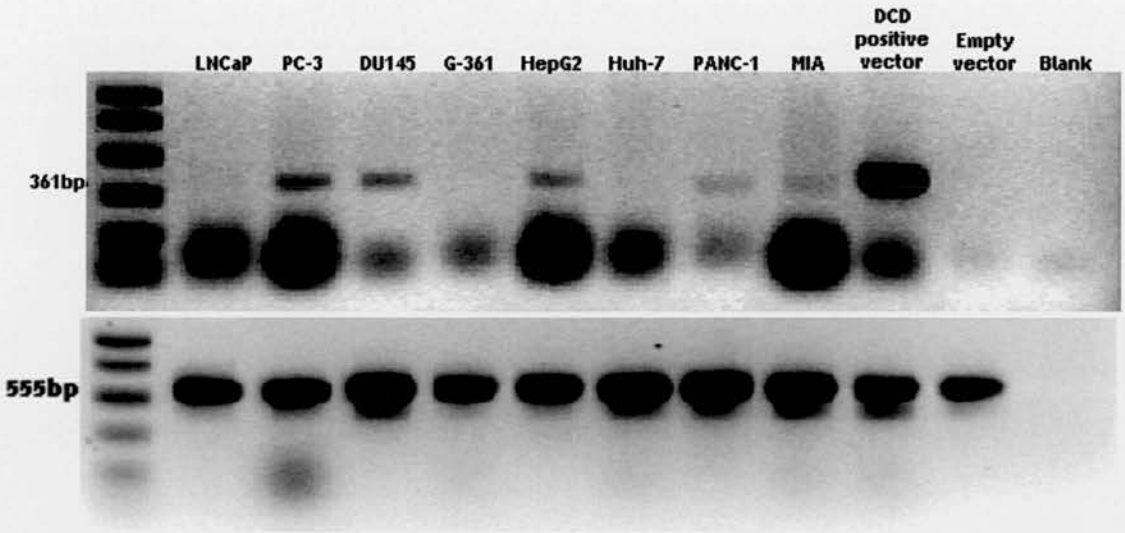

Figure 4.2 **RT-PCR of immortalised human cancer cell lines using *HCAP* (upper gel) and *MIC* (lower gel) primers.**
DCD cDNA product at 361bp. Bands were demonstrated for PC-3, DU145, Hep G2, PANC-1, MIA-Pa-Ca-2, PC-3M (not shown) and CFPAC (not shown) at the same level as DCD positive control. β_2-microglobulin cDNA product at 555bp present for all cell lines.

4.5.2 Nucleotide sequencing of PCR products

The 174bp and 361bp PCR products generated from the *PIF* and *HCAP* primers, respectively, were sent for nucleotide sequencing to confirm their identity. DCD cDNA was confirmed in products amplified by the *PIF* primers from PC-3, PC-3M, DU145, G-361, Hep G2, CFPAC, and MIA-Pa-Ca-2 cells, as well as from the prostate cell line PC-3 amplified with the *HCAP* primers. In all cases, the sequences obtained were those of the appropriate sections of the DCD cDNA nucleotide sequence that the primers were designed to amplify. Furthermore, in all cases, the nucleotide sequences were identical, and they exactly matched that of DCD cDNA (see Figure 2.1, p.181, and Figure 2.2, p.183). Moreover, the sequences all matched exactly with the sequence obtained from RT-PCR of the DCD-PC-3M positive control. The region of the DCD cDNA which corresponds to the PIF-CP cDNA also had an identical sequence in all of the positive cell lines and positive control, and it exactly matched the published sequence of the putative human homologue of PIF [204]. There were no polymorphic forms with mutations at the sites of the putative PIF *N*- and *O*-glycosylation sites, which would have made glycosylation more likely to occur.

PANC-1 cells produced only a weak band using the PIF primers and it was not possible to obtain a nucleotide sequence from the corresponding PCR product. Similarly, the DCD nucleotide sequence was confirmed positively in only 1 of the 6 cell lines (namely, PC-3) in which the *HCAP* primers amplified a product. Reasons for failure of sequencing might have included low cDNA concentration or inappropriateness of the PCR primers for sequencing. The fact that the amplified

products were of the correct size, and the fact that no other recognisable cDNA sequences were identified in these products means that it is highly likely that they did indeed represent DCD cDNA.

4.6 Discussion

DCD mRNA was expressed by multiple immortalised human cancer cells, but there were some minor differences from the previous results in Chapter 3, possibly due to clonal variation within cell lines. For example, LNCaP, which demonstrated marginal DCD mRNA expression on real-time PCR (C_T of 39.2), did not demonstrate expression with either the *PIF* or *HCAP* primers on RT-PCR, whereas PC3-M, which did not demonstrate expression on real-time PCR (C_T of 40), demonstrated expression with both the *PIF* and *HCAP* primers on RT-PCR. However, in most cell lines, DCD cDNA was identified by both sets of primers utilised. Sequencing of the PCR products was consistently possible only from the *PIF* primers. However, all sequenced PCR products revealed a sequence identical to that described as the potential human homologue of PIF [204]. Importantly, both primers encompassed the 90-nucleotide sequence of PIF-CP. These PCR results indicate that production of both the 110 amino acid DCD polypeptide and the 30-amino acid PIF-CP is possible from these cell lines. However, despite the theoretical production of PIF mRNA by human cancer cells, there is no evidence that the transcribed product is translated or that any ultimate protein is capable of inducing muscle proteolysis. Furthermore, the results of Chapter 3 would imply that any expression that does occur is at only a very low or unreliable level.

Glycosylation of the PIF-CP has been shown to be crucial for the increased muscle proteolysis induced by PIF in mice [203] (i.e. the non-glycosylated peptide alone is devoid of proteolytic activity). To date, there have not been any studies demonstrating that a homologue of glycosylated PIF can be produced in human

cells. Indeed, Monitto *et al* were only able to detect an unglycosylated secreted protein on stable forced expression of human PIF in multiple murine and human cell lines [204]. This group also found that tumour xenografts engineered to overexpress human PIF protein did not induce cachexia *in vivo* [204]. Bioinformatic analysis has suggested that the human PIF-CP is unlikely to be found in a glycosylated form, based on the predicted amino acid sequence [206]. Furthermore, in the present study, the lack of mutant or polymorphic species of DCD cDNA with a higher chance of successful glycosylation means that the existence of a functional human PIF remains doubtful.

In summary, based on the cumulative data from both Chapters 3 and 4, although DCD may be expressed by a subset of pancreatic tumours, analysis of pancreatic human cell lines suggests that the possibility of PIF glycosylation is unlikely. In OGC and prostate cancer, expression of DCD mRNA (and, hence, presumably PIF) is very low or, in most cases, absent. Thus, within upper GI cancer, a disease commonly associated with cancer cachexia in humans, the role of PIF as a mediator of skeletal muscle wasting should be viewed with caution.

In the next chapter, the area of investigation moves away from PIF to another potential tumour-derived mediator of skeletal muscle wasting, MIC-1.

Chapter 5 – Tumour-Derived Mediators III: Plasma MIC-1 correlates with systemic inflammation but is not an independent determinant of nutritional status or survival in oesophago-gastric cancer

5.1 Introduction

Systemic inflammation has been linked with adverse survival in a variety of cancer types [69, 78, 84]. This association could be explained by a variety of tumour-related phenomena including enhanced tumour progression [78], angiogenesis [548], and metastasis [549]. However, the presence of systemic inflammation has also been linked with both hypermetabolism [550] and reduced food intake [8], two key components of the cachexia syndrome known to result in shortened survival in patients with advanced malignancy [8].

The mechanisms whereby systemic inflammation arises in cancer patients are not established clearly. One hypothesis is that interaction between host and tumour cells within the tumour mass results in activation of TAMs [70] (see Chapter 1.4.1.1, p.44). The latter circulate to distant target organs where enhanced cytokine/mediator production results in the generation of a systemic inflammatory response. Target organs include the liver (production of acute phase proteins e.g. CRP) [70], the brain (induction of anorexia) [551] and skeletal muscle (induction of protein degradation and net amino-acid mobilisation) [225, 321]. Increased pro-inflammatory cytokine release by PBMCs from patients with elevated plasma CRP concentrations has been demonstrated previously [70]. The ability of such PBMCs

to induce acute-phase protein production from co-cultured human hepatocytes appeared to be IL-6-dependent [70]. In patients with OGC (where systemic inflammation is associated with weight loss and shortened survival), it has been shown previously that tumour IL-1β overexpression and chronic inflammatory cell infiltrate are independent factors influencing systemic inflammation [16]. However, the precise role of various pro-inflammatory cytokines within human tumours in the generation of a systemic response is still understood incompletely.

MIC-1 is a divergent member of the TGF-β superfamily that is produced by macrophages in response to activation (see Chapter 1.7.1.5, p.87) [552]. MIC-1 is not expressed in most human tissues at basal conditions (except the placenta) but is expressed at high concentrations during inflammation and injury [553, 554]. MIC-1 is overexpressed by malignant melanoma cells and is associated with tumourigenicity [555]. High serum concentrations of MIC-1 have been observed in patients with pancreatic [556, 557], gastric [558] and breast cancer [559], and have been associated with adverse survival in colorectal cancer [560] and glioblastoma [561]. In prostate cancer, high patient serum levels of MIC-1 have been associated with increased disease stage [562], docetaxel resistance [563] and adverse survival [564]. In prostate cancer bone metastases, MIC-1 induced osteoclast activation [565]. In mice bearing human prostate cancer xenografts, elevated MIC-1 concentrations were associated with marked weight, fat and lean tissue loss that was mediated by decreased food intake and was reversed by an antibody to MIC-1 [254]. Additionally, normal mice administered systemic MIC-1, and transgenic mice overexpressing MIC-1, demonstrated hypophagia and reduced body weight

[254]. In a small group of cachectic prostate cancer patients (n=26), serum MIC-1 concentrations were significantly associated with weight loss and weakly correlated with serum IL-6 [254]. Thus, a synthesis of the current data would suggest that MIC-1 might play a role in the aetiology of systemic inflammation, whereas animal data would imply a role in the induction of anorexia. The two roles are consistent, as many pro-inflammatory cytokines are known to induce centrally an anorectic state.

To determine whether MIC-1 is associated with human cachexia, this study aimed to measure circulating concentrations of MIC-1 in a large cohort (n>250) of patients with OGC, a disease strongly associated with cachexia, and to analyse the relationships between MIC-1, systemic inflammation, nutritional status, food intake and survival. MIC-1 was chosen as a mediator of interest, as it was believed initially that MIC-1 might provide a crossover link between host-derived and tumour-derived mediators within the host-tumour interaction (see Chapter 1.4.2, p.51). This is because the published data, to date, would suggest that the cellular source of MIC-1 might be either tumour cells and/or TAMs within the heterogenous tumour mass (Figure 5.1).

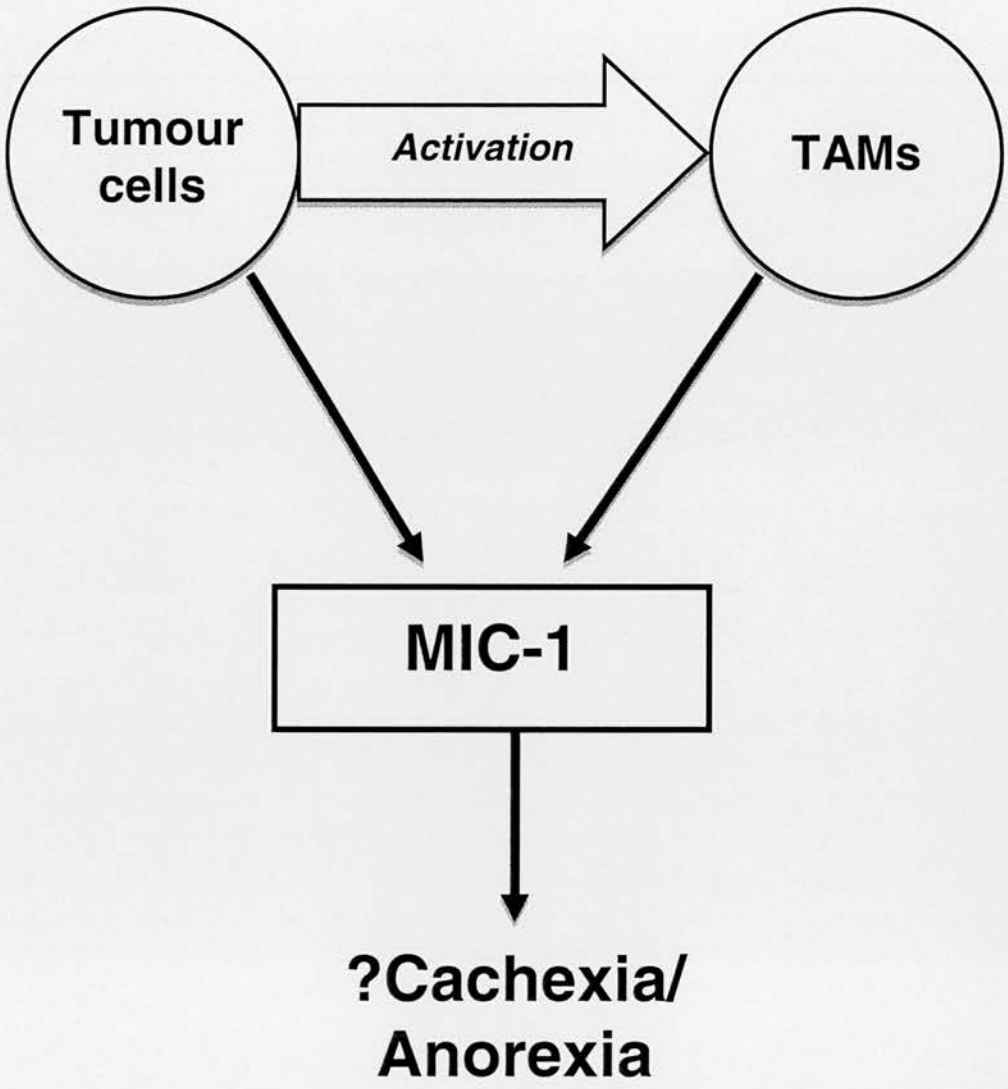

Figure 5.1 **The possible cellular origins of macrophage inhibitory cytokine-1 within the tumour mass.**
Within the tumour mass, macrophage inhibitory cytokine-1 (MIC-1) could be secreted by either the tumour cells and/or host tumour-associated macrophages (TAMs) activated as part of the host-tumour interaction.

5.2 Hypothesis

Plasma MIC-1 levels are elevated in patients with OGC and are associated with systemic inflammation, nutritional depletion, decreased food intake, and worsened survival.

5.3 Patients and healthy controls

Patients with a new histological diagnosis of OGC were recruited (n=293; 198 males and 95 females) (see Methods Chapter 2.1, p.167)[12]. Whole blood was taken at diagnosis for plasma analysis of MIC-1 (see Methods Chapter 2.3.6, p.172) and CRP concentration (see Methods Chapter 2.3.2, p.170). In a subset of patients (n=197), plasma albumin concentration was determined (see Methods Chapter 2.3.3, p.170) as well as CRP, and thus calculation of the systemic inflammation-based score, the mGPS [566], was performed. Nutritional assessment was also performed (see Methods Chapter 2.2, p.168). In a subset of patients (n=188), dysphagia score and subjective diet score were assessed (see Methods Chapter 2.2.6, p.169) as surrogate measures of food intake. The majority of patients (n=186) were followed up until death.

Thirty-five (25 males, 10 females) control subjects, including laboratory and hospital staff (n=28) and patients undergoing minor operative procedures for benign, non-inflammatory conditions (n=7), were recruited for comparative MIC-1 analysis (see Methods Chapter 2.1, p.167). Median age was 29yrs (range 24-85).

5.4 Statistical analysis

Plasma MIC-1 data are presented as box plots. Mild outliers (MIC-1 concentration more than 1.5 times the interquartile range [IQR] above the 3rd quartile/below the 1st quartile) are represented as circles and extreme outliers (MIC-1 concentration more than 3 times the IQR above the 3rd quartile/below the 1st quartile) are

[12] I acknowledge Mr. Christopher Deans who recruited some of these patients.

presented as stars. For visual clarity, the y-axes are limited to a maximum MIC-1 concentration of 5000 or 8000pg/ml. Differences between the distribution functions of data for 3 or more subject groups were determined by Kruskal-Wallis test (KWT) (displayed in text and figure legends). Subsequent analysis to determine differences between any 2 groups was determined by Mann-Whitney test (MWT) (displayed on figures). Correlation analysis was performed using non-parametric Spearman's Rank Correlation Coefficient. Linear regression was used to investigate the relationship between CRP and MIC-1. Significance levels for the explanatory variables of interest were 0.10 to enter stepwise into the model. Survival analyses were performed on those patients followed until death using univariate Kaplan-Meier (KM) plots and a multivariate Cox Proportional Hazards Model. For construction of the latter model, treatment regimen was defined as either surgery with curative intent, radical chemo/radiotherapy with curative intent, chemo/radiotherapy with palliative intent, or nil.

5.5 Results

5.5.1 Patient demographics

Demographics, nutritional status and plasma concentrations of inflammatory mediators of the OGC patients (n=293) are shown in Table 5.1. OGC patients exhibited a median weight loss of 6.4% (range 0.0-33.4%) (Table 5.1), and 34% of patients had lost ≥10% body weight, consistent with significant cachexia. Furthermore, median BMI, MAMC and TSF measures were lower than those reported in healthy elderly populations [567]. Forty two percent of patients (n=123) exhibited plasma CRP concentrations ≥10mg/L consistent with the presence of an APPR. Of those patients assessed, 29.9% (59/197) had a mGPS of 1 and 13.7% (27/197) had a mGPS of 2.

5.5.2 Plasma MIC-1 concentrations are elevated in oesophago-gastric cancer

Plasma MIC-1 was elevated in OGC patients (median 1371pg/mL; range 141-39053) compared with controls (median 377pg/mL; range 141-3786) ($p<0.001$) (Figure 5.2). Patients with gastric tumours (median 1592pg/mL; range 141-12643) demonstrated higher MIC-1 concentrations than patients with gastro-oesophageal junction (GOJ) (median 1337pg/mL; range 383-39053) and oesophageal tumours (median 1180pg/mL; range 258-31184) ($p=0.015$) (Figure 5.3). Patients with poorly-differentiated tumours (median 1480pg/mL; range 245-9000) demonstrated higher MIC-1 concentrations than patients with moderately-differentiated (median 1103pg/mL; range 378-6646) and well-differentiated tumours (median 875pg/mL; range 710-1407) ($p=0.010$) (Figure 5.4). Plasma MIC-1 concentration also

	Patients (n=293)
Male : Female	198 : 95
Age (yrs)	70 (26-91)
Tumour Site	
Oesophageal	139
Gastro-oesophageal junction	51
Gastric	103
Histology	
Adenocarcinoma	242
Squamous	43
Undifferentiated	6
Neuroendocrine	2
Disease Stage	
I	36
II	45
III	106
IV	97
Unknown	46
Body Mass Index (kg/m^2)	24.6 (13.9-46.7)
Weight Loss (% loss of pre-morbid weight)	6.4 (0.0-33.4)
Mid-Arm Muscle Circumference (cm)	23.8 (15.6-32.10)
Triceps Skinfold Thickness (mm)	12.0 (3.0-52.0)
Karnofsky Performance Score	90 (30-100)
CRP (mg/L)	9.0 (1.0-200.0)
MIC-1 (pg/mL)	1246.5 (140.7-39052.9)

Table 5.1 **Demographics and plasma concentrations of inflammatory mediators of the oesophago-gastric cancer patients.**
Although the median body mass index lay within the normal range, 34% of patients had lost ≥10% of pre-illness body weight. Data are presented as medians with ranges in parentheses.

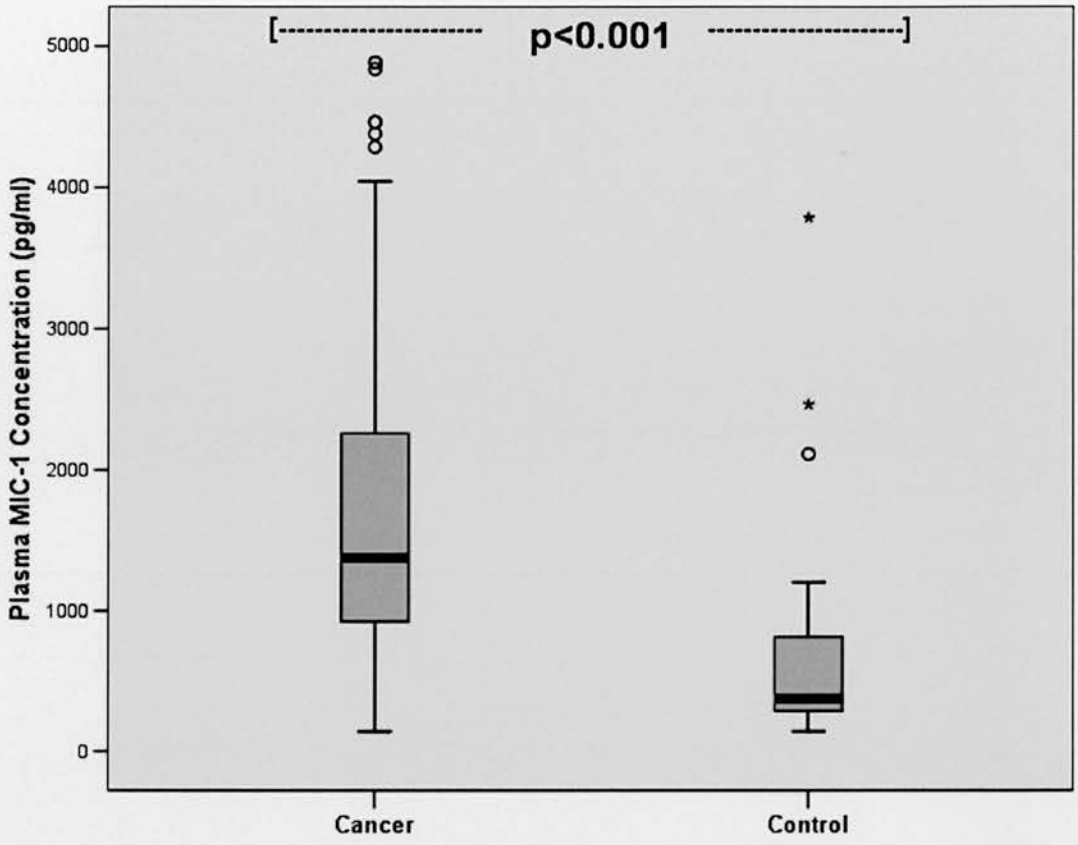

Figure 5.2 **Box plot demonstrating increased plasma MIC-1 concentration in cancer patients compared with controls (p<0.001).**
Circles and stars represent mild and extreme outliers, respectively.

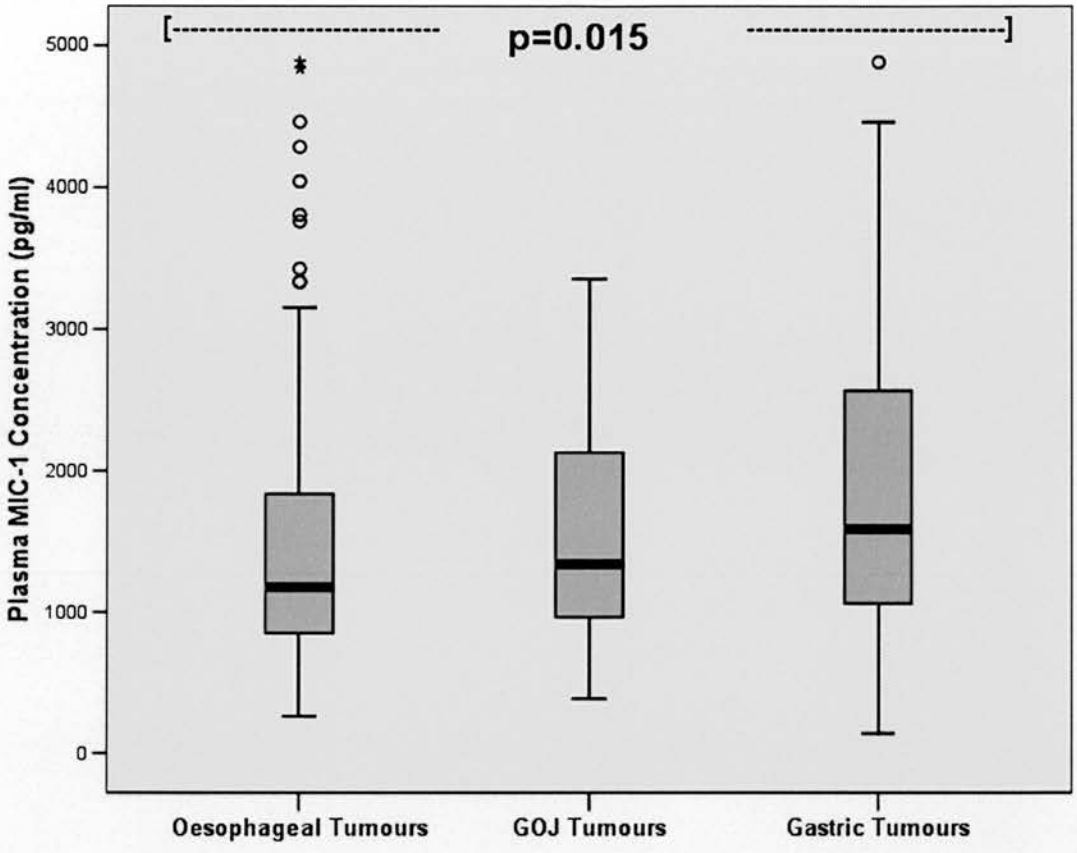

Figure 5.3 **Box plot demonstrating increasing plasma MIC-1 concentration with more distal situation of the primary tumour (p=0.015).**
Circles and stars represent mild and extreme outliers, respectively.
GOJ = gastro-oesophageal junction

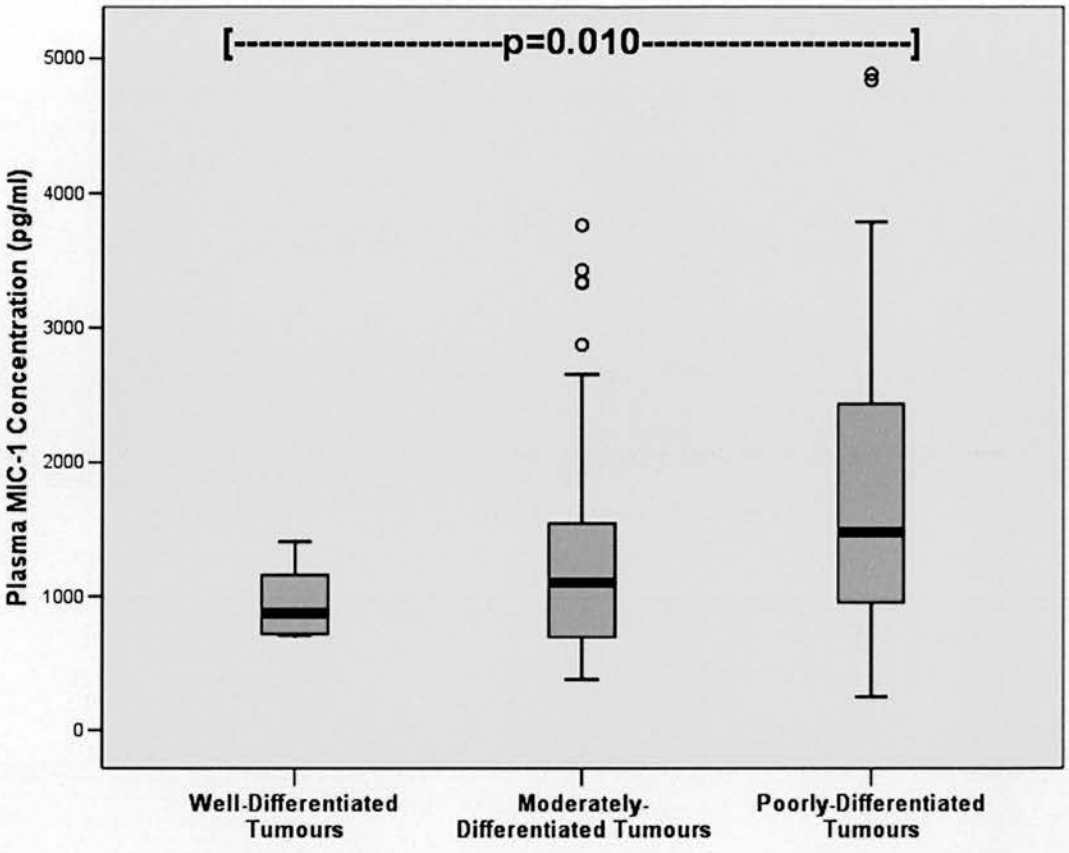

Figure 5.4 **Box plot demonstrating increasing plasma MIC-1 concentration with worsening tumour grade (p=0.010).**
Circles represent mild outliers.

increased with worsening disease stage (Figure 5.5) and increasing mGPS (Figure 5.6).

5.5.3 Relationship of MIC-1 with systemic inflammation and nutritional status

Plasma MIC-1 concentration correlated positively with disease stage ($r=0.217$; $p<0.001$), patient age ($r=0.332$; $p<0.001$), CRP ($r=0.314$; $p<0.001$) and mGPS ($r=0.336$; $p<0.001$), and correlated inversely with KPS ($r=-0.269$; $p<0.001$) (Table 5.2). Plasma MIC-1 also correlated weakly with diet score ($r=0.157$; $p=0.031$), but did not correlate with dysphagia score or any of the nutritional and anthropometric parameters measured. However, there was a small but significant increase (18.9%) in plasma MIC-1 between those patients who had lost $\geq$10% weight (median 1493pg/mL; range 258-31184) compared with those who had not (median 1256pg/mL; range 141-39053) ($p=0.036$). In contrast, CRP ($r=0.247$; $p<0.001$) and mGPS ($r=0.280$; $p<0.001$) both correlated with weight loss, and mGPS also correlated inversely with MAMC ($r=-0.318$; $p<0.001$), a measure of muscularity (Table 5.2). Furthermore, there was a highly significant increase in CRP and mGPS between those patients who had lost $\geq$10% weight compared with those who had not (median CRP 16.5mg/L vs 6.0mg/L; median mGPS 1 vs 0) ($p=0.001$ for both tests).

The relationship between MIC-1 and CRP was not linear, as a dot-plot demonstrated wide variance of CRP with increasing MIC-1 (Figure 5.7). However,

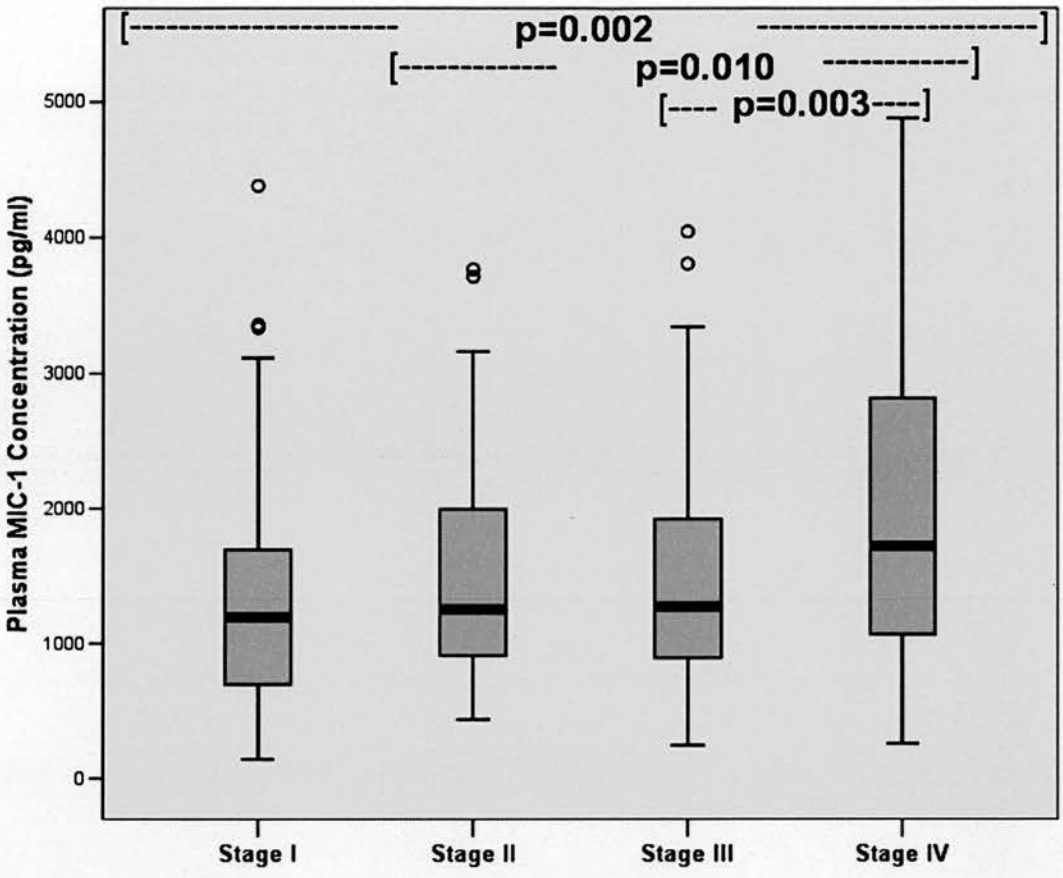

Figure 5.5 **Box plot demonstrating increasing plasma MIC-1 concentration with worsening disease stage (p=0.002).**
Circles represent mild outliers.

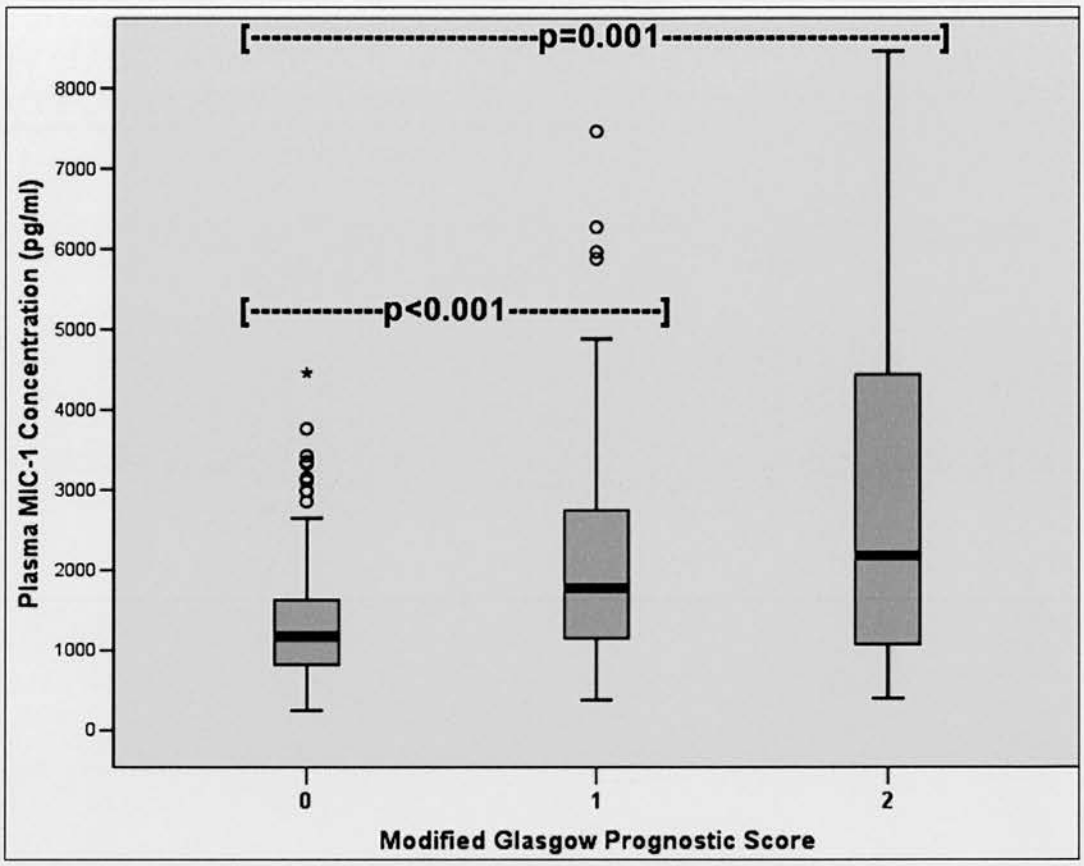

Figure 5.6 **Box plot demonstrating increasing plasma MIC-1 concentration with increasing mGPS (p<0.001).**
Circles and stars represent mild and extreme outliers, respectively.
mGPS = modified Glasgow Prognostic Score.

Inflammatory Mediator	Positive Correlates	r	*p*	Negative Correlates	r	*p*
MIC-1	CRP	0.314	*<0.001*	Albumin	-0.316	*<0.001*
	mGPS	0.336	*<0.001*	KPS	-0.269	*<0.001*
	Age	0.332	*<0.001*			
	Tumour Grade	0.234	*0.002*			
	Stage	0.217	*<0.001*			
	Diet Score	0.157	*0.031*			
CRP	MIC-1	0.314	*<0.001*	Albumin	-0.489	*<0.001*
	Tumour Grade	0.341	*<0.001*	KPS	-0.257	*<0.001*
	Stage	0.220	*<0.001*			
	% Weight Loss	0.247	*<0.001*			
	Diet Score	0.265	*<0.001*			
mGPS	MIC-1	0.336	*<0.001*	MAMC	-0.318	*<0.001*
	Tumour Grade	0.268	*0.001*	KPS	-0.362	*<0.001*
	Stage	0.234	*0.001*			
	% Weight Loss	0.280	*<0.001*			
	Diet Score	0.262	*<0.001*			

Table 5.2 **Correlations between inflammatory mediators and nutritional status in the oesophago-gastric cancer patients.** CRP and modified Glasgow Prognostic Score (mGPS) both correlated with percentage weight loss, whereas MIC-1 did not. Furthermore, mGPS correlated inversely with mid-arm muscle circumference (MAMC), a measure of muscularity. All three inflammatory mediators/scores correlated inversely with Karnofsky performance score (KPS), suggesting a negative impact on muscle function.

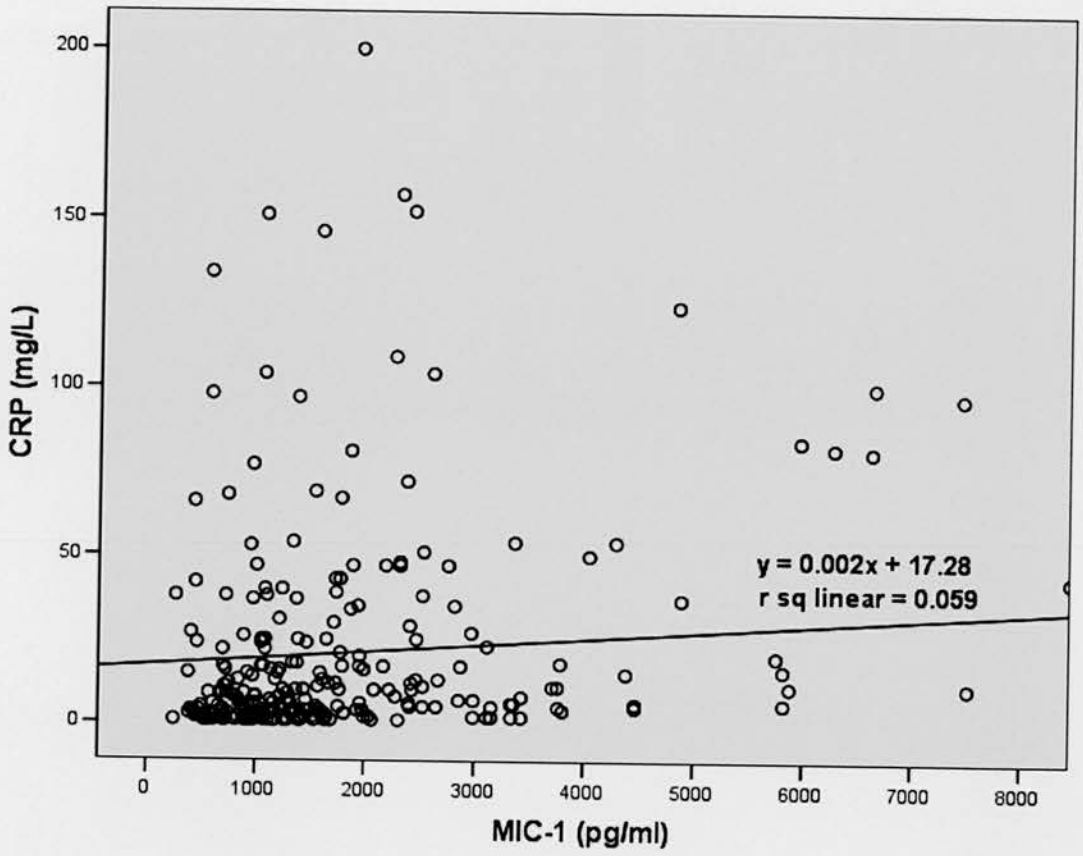

Figure 5.7 **Dot plot of MIC-1 versus CRP (linear $r^2 = 0.059$).**
There was wide variance of plasma CRP in relation to increasing plasma MIC-1. Assuming linearity, regression analysis demonstrated that plasma MIC-1 accounted for only 5.9% of the variation in plasma MIC-1.

assuming linearity, regression analysis demonstrated that MIC-1 accounted for 5.9% of the variation in plasma CRP ($p<0.001$). In a regression model of CRP (incorporating MIC-1, disease stage, tumour grade, percentage weight loss, diet score and KPS i.e. those factors found to correlate with CRP), plasma MIC-1 concentration ($p=0.003$), diet score ($p=0.001$), and tumour grade ($p=0.033$) were significant determinants ($r^2=0.179$) (Table 5.3). In a regression model of percentage weight loss (incorporating disease stage, mGPS, diet score and dysphagia score), all four factors were significant determinants ($r^2=0.259$) (Table 5.3). Plasma MIC-1 concentration did not qualify to enter the model.

5.5.4 Relationship of MIC-1 with survival

Patients with plasma MIC-1 concentrations in the upper quartile (>2270pg/mL) demonstrated worsened survival (median 204d; 95%CI: 157-251) compared with patients with MIC-1 concentrations in the lower 3 quartiles (median 316d; 95%CI: 259-373) ($p=0.036$; log rank test) (Figure 5.8). In a Cox Proportional Hazards model (incorporating MIC-1, CRP, percentage weight loss, disease stage, tumour grade, patient age, dysphagia score, diet score, and treatment regimen), disease stage ($p<0.001$), treatment regimen ($p=0.003$), CRP ($p=0.034$), and percentage weight loss ($p=0.002$), but not plasma MIC-1 concentration, were significant determinants of survival (Table 5.4). Substitution of mGPS for CRP within the model revealed stage, treatment regimen and weight loss as the only significant determinants.

Model	Factor	Unstandardised Coefficients		Standardised Coefficients	t	*p*	95% CI for B	
		B	Standard Error	Beta			Lower Boundary	Upper Boundary
CRP	(Constant)	-35.759	13.387		-2.671	*0.008*	-62.213	-9.305
	MIC-1	0.005	0.002	0.231	3.001	*0.003*	0.002	0.008
	Diet Score	12.613	3.670	0.258	3.437	*0.001*	5.361	19.864
	Tumour Grade	9.977	4.628	0.165	2.156	*0.033*	0.832	19.123
% Weight Loss	(Constant)	-3.788	2.107		-1.798	*0.074*	-7.946	0.370
	Diet Score	2.871	1.066	0.221	2.693	*0.008*	0.767	4.975
	mGPS	2.491	0.835	0.205	2.983	*0.003*	0.843	4.140
	Stage	1.459	0.639	0.161	2.284	*0.024*	0.198	2.719
	Dysphagia Score	1.182	0.583	0.155	2.028	*0.044*	0.031	2.333

Table 5.3 **Results of the regression models of CRP (r^2=0.179) and percentage weight loss (r^2=0.259) demonstrating significant determinants.**

Plasma MIC-1 was a weak but significant determinant of plasma CRP. Although several factors, including modified Glasgow Prognostic Score (mGPS), were found to be significant determinants of percentage weight loss, plasma MIC-1 did not even qualify to enter the model.

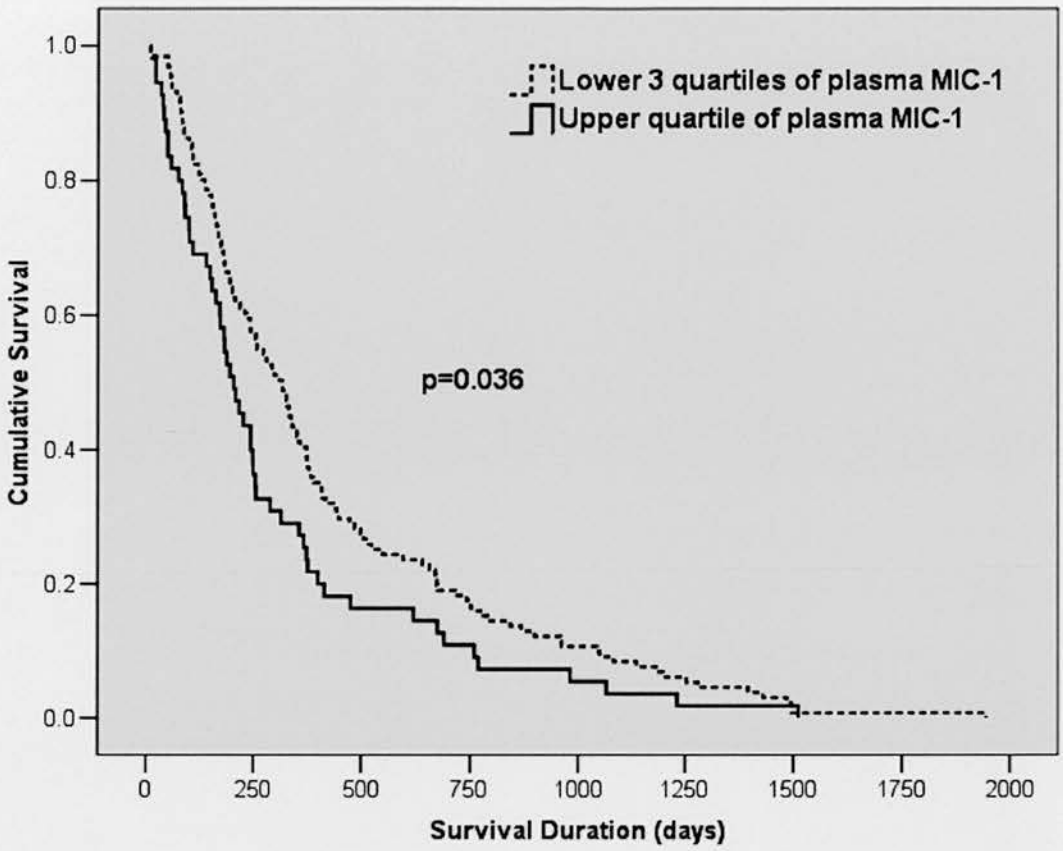

Figure 5.8 **Kaplan-Meier curve of survival of oesophago-gastric cancer patients according to plasma MIC-1 concentration.**
Patients with MIC-1 concentrations in the upper quartile exhibited worsened survival (median 204d; 95%CI: 157-251) compared with patients with MIC-1 concentrations in the lower 3 quartiles (median 316d; 95%CI: 259-373) (p=0.036, log rank test).

	B	SE	Wald	df	*p*	Hazard Ratio	95% CI	
							Lower Boundary	Upper Boundary
Stage	0.682	0.177	14.902	1	*<0.001*	1.979	1.399	2.798
Treatment Regimen			13.934	3	*0.003*			
CRP	0.006	0.003	4.498	1	*0.034*	1.006	1.000	1.012
% Weight Loss	0.038	0.013	9.232	1	*0.002*	1.039	1.014	1.065

Table 5.4 **Results of the Cox Proportional Hazards model demonstrating significant determinants of oesophago-gastric cancer patient survival.**
Significant determinants of patient survival included disease stage, treatment regimen, plasma CRP concentration and percentage weight loss, but not plasma MIC-1.

5.6 Discussion

The present study demonstrates that plasma MIC-1 concentrations are elevated in OGC patients compared with controls. Furthermore, increasing plasma MIC-1 concentrations are associated with indicators of poor patient prognosis, including tumour grade and stage. Elevated circulating concentrations of MIC-1 have also been associated with poor prognostic indicators in other cancer types, including prostate cancer [562, 564], colorectal cancer [560] and glioblastoma [561].

In the present study, plasma MIC-1 also correlated significantly with mGPS and plasma CRP concentration suggesting that MIC-1 might play a role in the aetiology of systemic inflammation in OGC (although linear regression implies that this may be a minor effect). In previous studies of advanced prostate cancer patients and same-sex twins, MIC-1 has been correlated with serum IL-6 concentration. IL-6 is a key regulator of the hepatic APPR during cancer cachexia [70], and therefore MIC-1-induced stimulation of IL-6 may be the underlying mechanism linking MIC-1 to elevation in CRP levels.

Plasma MIC-1 concentrations did not correlate with any measured nutritional or anthropometric parameters, including patient weight loss or measures of muscularity, such as MAMC. In a recent cohort of 220 patients with OGC, it was demonstrated by multiple regression analysis that plasma CRP, stage of disease and dietary intake were independent variables in determining degree of weight loss [515]. These results are confirmed by the present study. Given such importance of systemic inflammation, disease stage and dietary intake in the genesis of weight

loss in OGC patients, it is somewhat surprising that although MIC-1 concentrations correlated with all three of these variables in the current study, there was no correlation with weight loss. However, MIC-1 levels were slightly elevated in patients with ≥10% weight loss compared with patients without, suggesting that MIC-1 may play a role in the maintenance, rather than the initiation, of weight loss.

Studies in mice with xenografted prostate tumours have suggested that the mechanism underlying MIC-1-induced weight loss is hypophagia caused by reduced NPY expression and increased POMC expression in the hypothalamic ARC [254]. In patients with OGC, a number of primary and secondary causes of reduced dietary intake are at work simultaneously, including dysphagia [568], early satiety [569], chronic nausea [568], alterations in circulating neuroendocrine hormones (e.g. ghrelin [570] and leptin [571, 572]), hypogeusia/hyposmia [232], and cytokine-induced central anorexia [232, 573]. Assuming that MIC-1 may be a modulator of appetite in humans (there was a weak correlation between plasma MIC-1 with subjective diet score), the complex constellation of additional factors also controlling food intake may explain why plasma MIC-1 concentrations did not correlate with nutritional status in the present study. Future studies of MIC-1 that record accurately calorific intake in OGC patients are required to elucidate the anorectic effect of different factors.

Another proposed mechanism of MIC-1 induced weight loss is via paracrine effects on adipocytes [574]. Recombinant MIC-1 stimulates adiponectin secretion by human adipocytes, thus potentially negatively regulating body fat mass [574].

However, in the present study, MIC-1 did not correlate with a measure of adiposity, namely TSF. Furthermore, previous studies have proven that the relationship between MIC-1 and fat mass is clearly not understood fully, as both obesity and Type II DM are associated with increased circulating levels of MIC-1 [575]. However, the latter observation might be consistent with a role for MIC-1 in the aetiology of systemic inflammation.

An alternative hypothesis to explain the lack of association between plasma MIC-1 and nutritional status in OGC is that circulating MIC-1 concentrations are simply not elevated sufficiently to overcome regulatory mechanisms and induce cachexia. In patients with prostate cancer and cachexia, serum MIC-1 concentrations were significantly higher (mean 12416 ± SD 10235pg/mL) [254] than those measured in the present cohort of OGC patients. Furthermore, in mice xenografted with prostate tumours overexpressing human MIC-1, only animals with serum concentrations >8500pg/mL demonstrated clinically significant levels of weight loss [254]. Of the OGC patients in the present study, only 5 (1.7%) demonstrated plasma concentrations of MIC-1 >8500pg/mL. In these individuals, plasma CRP was also elevated (median 58mg/L; range 16-92) but there was no significant reduction in BMI (median 30.1kg/m^2; range 21.7–32.0) or increase in weight loss (median 6.5%; range 0.0-12.5) compared with the rest of the group. Reasons for low circulating MIC-1 concentrations in OGC patients may include low levels of activation of MIC-1 through p53-dependent mechanisms [576-578], as many oesophago-gastric tumours demonstrate p53 deletion, mutation and loss of heterozygosity [579, 580]. In the present study, elevated plasma MIC-1 was associated with worsened survival

on univariate but not multivariate analysis. This is in contrast to a recent study of 876 male subjects aged 35–80 years (selected from the Swedish Population Registry) and a cohort of 324 same-sex twins (69% female) (selected from the Swedish Twin Registry) that suggested that serum MIC-1 concentration can be used as a marker of all-cause mortality, even when data were adjusted for age, BMI and smoking history [581]. However, importantly, even though cancer accounted for 64 (23%) of the 276 deaths seen in these two cohorts, serum MIC-1 concentration did not correlate with BMI. Such findings may vary depending on the definition of normal MIC-1 concentration employed. Other studies which have analysed relatively large cohorts of healthy controls have suggested that the upper limit of normal plasma MIC-1 concentration lies between 1070–1600pg/mL [556, 557, 560] i.e. lower than the definition employed in the present study. However, the present data also suggest that plasma MIC-1 concentration may increase with patient age, thus implying that the normal range is not static. A lack of age-matched healthy controls could be considered a disadvantage of both previous studies and the present.

The present study did not investigate the exact cellular source of MIC-1 within the tumour mass. However, the variation in plasma MIC-1 concentrations observed between different tumour types, grades and stages implicates a tumour cell-specific mechanism in the induction of MIC-1 expression. Furthermore, previous researchers have found that MIC-1 is produced by tumour cells, rather than TAMs [253]. MIC-1 is often secreted in an unprocessed propeptide form (proMIC-1) that regulates the balance between ECM stores and circulating mature

MIC-1 [253]. The absence of propeptide in xenograft animal tumour models is associated with a 20-fold increase in serum MIC-1 [253]. In low-grade localised prostate cancer, the level of proMIC-1 stromal stores was the best predictor of future disease relapse compared with other clinicopathological variables [253]. Mechanisms surrounding the processing and extracellular storage of MIC-1 may represent one explanation for the observed differences in plasma MIC-1 between different tumour variables. Therefore, it seems likely that MIC-1 is secreted predominantly by tumour cells. Further work targeted specifically at host mononuclear cells is required if evidence of TAM production of MIC-1 is to be proven. The distinct role of macrophages in tumor progression and suppression is influenced by tumour microenvironment [41]. Thus, dependent on the nature of the trigger, macrophage-secreted MIC-1 might play an important role in the host-tumour microenvironment to decide the fate of the cancer.

In conclusion, although plasma MIC-1 correlates with tumour grade, disease stage, dietary intake and systemic inflammation, it does not appear to mediate weight loss, nutritional depletion or muscle wasting significantly in OGC. Thus, MIC-1 is unlikely to be a useful biomarker of skeletal muscle wasting in clinical trials.

In Chapters 3 to 5, this thesis has concentrated on the investigation of possible tumour-derived mediators of cachexia. MIC-1 was proposed initially, although not proven, as a mediator that may cross the boundary between tumour-derived and host-derived circulating mediators. In Chapter 6, attention turns away from tumour-

derived mediators and focuses solely on the study of other host-derived circulating mediators, namely sex steroids and gonadotropins.

Chapter 6 Neuroendocrine Mediators: Interaction of gonadal status with systemic inflammation and opioid use in determining nutritional status and prognosis in advanced pancreatic cancer

6.1 Introduction

Patients with advanced pancreatic cancer survive only 3-6 months and have a high incidence of cachexia [582]. Epidemiological studies suggest that prognosis may be worse for older men and younger women [582]. Mechanisms underlying these observations are unclear but may reflect interactions between pro-inflammatory cytokines, sex hormones and nutritional status, all of which might be subject to intervention, thus improving prognosis.

A key determinant of prognosis in pancreatic cancer is systemic inflammation [69], a core component of the complex syndrome of cachexia [8]. Patients with pancreatic cancer exhibit elevated circulating IL-6, TNF-α and sTNFR75 [518], and pancreatic tumours demonstrate upregulation of IL-6 in association with systemic inflammation [583].

Hypogonadism is common in elderly men (i.e. the age group most at risk of pancreatic cancer), with an incidence of approximately 20% in males aged 60 to 80yrs old [584]. Androgens are key anabolic regulators of skeletal muscle mass and the relative lack of androgens in hypogonadal patients has been hypothesised as one

cause of age-related skeletal muscle atrophy [585]. Thus, within cancer cachexia, hypogonadism is a possible contributor to muscle wasting [585], weight loss and, potentially, accelerated demise. Male patients with metastatic malignancy, including pancreatic cancer, demonstrate low circulating testosterone [101, 586]. Furthermore, such patients often receive opioid analgesia, an iatrogenic cause of secondary hypogonadism [587]. One study of male cancer patients demonstrated that plasma levels of free testosterone and bioavailable testosterone correlated inversely with plasma IL-6 [586], suggesting a potential interplay between pro-inflammatory cytokines and the hypothalamic-pituitary-gonadal axis.

In female pancreatic cancer patients, elevated oestradiol concentrations have been observed [588]. There exists a complex relationship between oestrogens and systemic inflammation. For example, the exogenous use of oestrogens by healthy post-menopausal women increases systemic inflammation [589]. Furthermore, pro-inflammatory cytokines stimulate aromatase activity in peripheral tissues (e.g. fat) in post-menopausal women [590]. However, the association between oestradiol and cytokines in female cancer patients has not been studied.

The aim of the present study was to investigate the interaction of circulating sex steroid and gonadotropin levels with nutritional status, systemic inflammation and survival in males and females with advanced pancreatic cancer. Furthermore, as a recognised aetiological factor in hypogonadism, the impact of opioid use was also investigated.

6.2 Hypothesis

Serum testosterone levels are reduced in male pancreatic cancer patients and are reduced further by opioid use, whereas serum oestradiol levels are elevated in female pancreatic cancer patients. Alterations in the levels of circulating sex steroids are associated with nutritional depletion, systemic inflammation, and worsened survival.

6.3 Patients and healthy controls

Two hundred patients with histologically proven or a firm radiological or operative diagnosis of unresectable pancreatic cancer (see Methods Chapter 2.1, p.167), of whom 176 had blood drawn for hormonal status (see Methods Chapter 2.3.4, p.171), serum CRP concentration (see Methods Chapter 2.3.2, p.170), serum albumin concentration (see Methods Chapter 2.3.3, p.170), and serum IL-6 and TNF-α concentrations (see Methods Chapter 2.3.5, p.172), were recruited originally to a multicentre, randomised trial comparing a protein and energy-dense nutritional supplement enriched with n-3 fatty acids (EPA) and antioxidants with an isocaloric isonitrogenous control supplement [406][13]. Patients were included if they had lost weight over the previous 6 months, had a KPS ≥60, and had a life expectancy greater than 2 months. Patients were excluded if they had undergone surgery, endoscopic stenting, radiotherapy, or chemotherapy during the previous 4 weeks, or had other active medical conditions (e.g. major GI disease, CRF, uncontrolled DM, HIV). At the time of study, no patient had gross ascites or oedema, jaundice, pyrexia, severe anaemia, or clinical or radiological evidence of infection. Nutritional assessment was performed (see Methods Chapter 2.2, p.168) and LBM was measured using BIA (see Methods Chapter 2.2.2, p.168). Body composition results have been published previously [406]. Median survival from enrolment was 130 days, with no significant difference between groups (experimental 142d vs control 128d). Thus, for the purposes of survival analysis, treatment of patients during follow-up can be considered uniform.

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[13] I acknowledge Abbott Laboratories, Chicago, IL, USA, and the numerous investigators of the Cancer Cachexia Study Group, who recruited these patients to the original randomised trial.

In the present study, blood and nutritional parameters were measured prior to nutritional supplementation. Premenopausal women (age <50yrs with serum oestradiol >150pmol/L or FSH <30U/L) were excluded retrospectively (n=1). Patients receiving glucocorticoids (n=7 patients receiving dexamethasone) or sex hormones (n=1 patient receiving medroxyprogesterone) were excluded. The final study cohort therefore consisted of 167 patients (90 males and 77 post-menopausal females). Four (2.3%) patients were taking thyroxine for hypothyroidism, and 26 (14.9%) were taking hypoglycaemics for the management of DM, of whom 17 (9.7%) were self-administering insulin. Most patients (n=147; 84.0%) were followed until death. The remainder were followed until study completion (8 weeks) or dropout [406].

Opioid use was defined as 'regular' or 'as required' controlled opioids, including oral morphine sulphate, oxycodone, hydromorphone, or transdermal fentanyl patch. Dihydrocodeine, a weak opioid, and tramadol, an atypical opioid that has limited activity through the opioid receptor, were not analysed. Oral morphine-equivalence per 24hrs was calculated according to standard conversion tables published in the British National Formulary [591]. Twenty-two (88%) of the 25 opioid-using males with pancreatic cancer received a regular daily dose plus an additional undefined, 'as required' dosage for breakthrough pain. The remaining 3 patients only took 'as required' opioids. Thus, it was not possible to assess average total daily dosage due to the variability in 'as required' opioid use. For the purposes of statistical analysis, regular daily oral morphine-equivalent dose was used as the tested variable.

Nineteen (10 males, 9 females) elderly, non-cancer control subjects were recruited to support and verify hormonal analyses[14] (see Methods Chapter 2.1, p.167). Median age (69yrs; range 56-86) was not different from the cancer patients (p=0.697).

6.4 Statistical analysis

Data are presented as medians with ranges. MWT and Fisher's Exact test (FET) were used to compare variables between different subject groups. Correlation analysis was performed using non-parametric Spearman's Rank Correlation Coefficient in an exploratory fashion and correction was not made for multiple comparisons. When investigating any potential correlation between opioid use and serum sex steroid/gonadotropin concentrations, regular daily oral morphine-equivalent dose was used as the tested variable. Univariate analysis of variance (ANOVA) was used to investigate the effect of covariates on dependent variables and to establish effect size using partial eta-squared test (Levene's test for equality of variance $p>0.05$). Survival analyses were performed using univariate KM plots and multivariate Cox Proportional Hazards models.

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[14] I acknowledge Mr. Alastair Moses who recruited these healthy controls.

6.5 Results

6.5.1 Patient sex steroid and gonadotropin status

Demographics, disease characteristics and nutritional status of the cancer patients (n=167) are shown in Table 6.1. All patients reported weight loss. Sex steroid, gonadotropin, CRP and pro-inflammatory cytokine levels of cancer patients and elderly controls are shown in Table 6.2. TT was reduced in male patients (median 12.4nmol/L; range 0.3-33.5) compared with male controls (median 16.9nmol/L; range 8.3-27.2) (p=0.045). Using TT definition, 48% of male patients were eugonadal, whereas 9% had early primary hypogonadism (normal TT and raised LH), 2% had frank primary hypogonadism (low TT and raised LH), and 37% had secondary hypogonadism (low TT and low/normal LH). Thus, 48% of male patients were hypogonadal, compared with 30% of controls (NS). Using cFT definition, 73% of male patients were hypogonadal compared with 40% of controls (p=0.029).

SHBG was elevated in male patients (median 65.7nmol/L; range 11.7-335.0) compared with controls (median 41.5nmol/L; range 23.0-93.0) (p=0.030). Similarly, SHBG was elevated in female patients (median 89.5nmol/L; range 35.0-232.0) compared with controls (median 49.0nmol/L; range 28.0-98.0) (p=0.001). Oestradiol also demonstrated a trend towards elevation in female patients (median 75.0pmol/L; range 28.0-2070.0) compared with controls (median 58.0pmol/L; range 36.0-133.0) (p=0.069). Eighteen percent of female patients exhibited high/premenopausal levels of oestradiol, compared with none of the controls (NS).

	Male (n=90)	Female (n=77)	*p*
Age (yrs)	67 (43-94)	69 (50-91)	*0.080*
Disease Stage			*0.872*
I	6	0	
II	31	35	
III	18	15	
IV	32	25	
Unknown	3	2	
Weight (kg)	66.1 (41.5-94.6)	55.0 (32.7-86.0)	*<0.001*
Body Mass Index (kg/m^2)	21.8 (13.6-30.0)	22.0 (13.6-33.2)	*0.890*
Lean Body Mass (kg)	49.5 (35.6-64.1)	36.6 (28.8-49.5)	*<0.001*
% Weight Loss (% loss of pre-morbid weight)	15.1 (1.6-35.0)	16.5 (4.7-40.1)	*0.269*
Grip Strength (kg)	33.0 (10.0-50.0)	19.0 (6.0-35.0)	*<0.001*
CA19.9 (kU/L)	734.0 (0.7-168100.0)	655.0 (0.5-716000.0)	*0.601*

Table 6.1 **Demographics, disease stage, nutritional status and tumour marker status of the pancreatic cancer patients.**
Although the median body mass index lay within the normal range for both sexes, all patients reported weight loss compared with pre-illness weight. Data are presented as median values with ranges in parentheses.

	Male			Female		
	Cancer (n=90)	**Control (n=10)**	***p***	**Cancer (n=77)**	**Control (n=9)**	***p***
Total Testosterone Male 10-30 nmol/L Female 0.4-2.8 nmol/L	12.4 (0.3-33.5)	16.9 (8.3-27.2)	*0.045*	1.0 (0.4-59.0)	N/A	N/A
Calculated Free Testosterone Male >0.245 nmol/L	0.162 (0.005-0.568)	0.278* (0.154-0.422)	*0.005*	0.008 (0.003-0.864)	N/A	N/A
Oestradiol Male <200 pmol/L Postmenopausal Female <150 pmol/L	N/A	N/A	N/A	75.0 (28.0-2070.0)	58.0 (36.0-133.0)	*0.069*
Sex hormone-binding globulin Male 6-45 nmol/L Female 30-120 nmol/L	65.7 (11.7-335.0)	41.5 (23.0-93.0)	*0.030*	89.5 (35.0-232.0)	49.0 (28.0-98.0)	*0.001*
Luteinising hormone Male 1.5-9.0 U/L Postmenopausal Female >20 U/L	4.4 (0.3-39.7)	3.9 (1.7-9.4)	*0.705*	20.8 (0.9-44.9)	19.7 (11.0-44.6)	*0.712*
Follicle-stimulating hormone Male 1.5-9.0 U/L Postmenopausal Female >30 U/L	6.4 (0.7-55.1)	6.1 (3.4-14.0)	*0.696*	61.6 (1.0-130.2)	49.9 (29.8-95.9)	*0.671*
CRP Normal <10mg/L	9.8 (0.8-356.9)	1.8 (0.9-9.3)	*0.003*	9.7 (0.9-337.4)	1.3 (0.9-2.8)	*0.002*
Interleukin-6 Normal 1.3+/-3.2 pg/mL	7.1 (1.3-201.0)	3.2 (2.0-9.6)	*0.007*	5.0 (1.5-127.7)	2.4 (1.1-30.3)	*0.006*
Soluble TNF receptor 75 Normal 5.2+/-2.1 pg/mL	12.0 (3.4-994.0)	6.3 (0.0-19.9)	*0.001*	13.2 (3.7-58.9)	4.6 (2.8-15.0)	*<0.001*

Table 6.2 **Serum sex hormone and pro-inflammatory mediator levels of the pancreatic cancer patients and controls.** Total testosterone and calculated free testosterone were significantly lower in male cancer patients compared with controls, whereas oestradiol levels demonstrated a trend towards elevation in female cancer patients compared with controls. Inflammatory mediators, including CRP, IL-6 and sTNFR75 were elevated in cancer patients of both sexes compared with sex-matched controls. Data are presented as median values with ranges in parentheses. *Based on a control serum albumin level of 42.5 g/L.

Median LH and FSH levels did not differ significantly between cancer patients and controls for either sex.

6.5.2 Systemic inflammation

In both sexes, CRP, IL-6 and sTNFR75 concentrations were higher in cancer patients compared with controls ($p<0.01$) (Table 6.2). Forty nine percent (n=81) of cancer patients exhibited an APPR (CRP $\geq$10mg/L) compared with none of the controls ($p<0.001$).

6.5.3 Interactions between gonadal status, systemic inflammation and nutritional status

In male patients, cFT correlated positively with BMI ($r=0.349$, $p<0.001$) and grip strength ($r=0.229$, $p=0.034$), and correlated inversely with percentage weight loss ($r=-0.287$, $p=0.007$), CRP ($r=-0.426$, $p<0.001$) and IL-6 ($r=-0.303$, $p=0.004$). Using cFT definition, hypogonadal male patients demonstrated greater percentage weight loss (16.7% vs 11.3%; $p=0.007$) and higher CRP (12.2mg/L vs 3.9mg/L; $p=0.001$), IL-6 (8.2pg/mL vs 4.5pg/mL; $p=0.015$) and sTNFR75 (14.2pg/mL vs 11.6pg/mL; $p=0.034$) compared with eugonadal patients, but did not differ significantly in age (67yrs vs 66yrs; $p=0.917$) or disease stage ($p=0.298$). In univariate ANOVA models of TT ($r^2=0.273$) and cFT ($r^2=0.309$) (incorporating CRP, opioid dosage, BMI, LH, FSH, age and disease stage as covariates), CRP (TT: $p=0.004$, effect size 10.2%; cFT: $p=0.007$, effect size 9.0%), opioid dosage (TT: $p=0.007$, effect size 8.7%; cFT: $p=0.009$, effect size 8.6%) and BMI (cFT: $p=0.005$, effect size 9.7%) were significant determinants.

In female patients, the relationship between sex steroid and inflammatory mediator levels was reversed. Oestradiol correlated positively with sTNFR75 (r=0.299, p=0.008) and demonstrated a trend towards a positive correlation with CRP (r=0.210, p=0.066) and IL-6 (r=0.193, p=0.091). Female patients with high/premenopausal levels of oestradiol demonstrated higher CRP (28.9mg/L vs 9.0mg/L; p=0.010), IL-6 (10.0pg/mL vs 4.6pg/mL; p=0.025) and sTNFR75 (18.6pg/mL vs 13.1pg/mL; p=0.040) compared with eugonadal patients, but did not differ significantly in age (71yrs vs 69yrs; p=0.687) or disease stage (p=0.659). In a univariate ANOVA model of oestradiol (r^2=0.941), CRP was the only significant determinant (p<0.001, effect size 67.9%).

SHBG correlated inversely with nutritional status in both sexes, and positively with systemic inflammation in male patients. In this latter group, SHBG correlated inversely with weight (r=-0.326, p=0.002), BMI (r=-0.261, p=0.013), LBM (r=-0.220, p=0.047) and albumin (r=-0.309, p=0.004), and positively with IL-6 (r=0.285, p=0.007) and sTNFR75 (r=0.227, p=0.033). In female patients, SHBG correlated inversely with weight (r=-0.233, p=0.042) and BMI (r=-0.234, p=0.041), and positively with percentage weight loss (r=0.265, p=0.020) and IL-6 (r=0.231, p=0.043).

6.5.4 Relationship between male hypogonadism or female "hyperoestrogenism" and survival

Using KM analysis and TT definition, hypogonadal male patients demonstrated worsened survival (median 75.0d; 95% confidence interval [CI] 57.3-92.8)

compared with eugonadal male patients (median 140.0d; 95%CI 95.9-184.1) ($p<0.001$, log rank test) (Figure 6.1). Similarly, using cFT definition, hypogonadal male patients demonstrated worsened survival (median 83.0d; 95%CI 66.9-99.1) compared with eugonadal male patients (median 153.0d; 95%CI 60.5-245.5) ($p=0.005$) (Figure 6.2).

As expected, presence of an APPR was also associated with worsened survival ($p=0.013$). When hypogonadism, presence of an APPR, percentage weight loss, disease stage and patient age were used as variables in a Cox proportional hazards model, the hazards ratio (HR) of death associated with male hypogonadism using TT definition was 2.87 (95%CI 1.73-4.75; $p<0.001$), and using cFT definition was 2.26 (95%CI 1.21-4.24; $p=0.011$).

Female patients with elevated/premenopausal serum levels of oestradiol demonstrated worsened survival (median 52.0d; 95%CI 29.9-74.1) compared with female patients who had normal post-menopausal levels of oestradiol (median 163.0d; 95%CI 114.8-211.2) ($p=0.020$) (Figure 6.3). When hyperoestrogenism, presence of an APPR, percentage weight loss, disease stage and patient age were used as variables in a Cox proportional hazards model, the HR of death associated with elevated oestradiol was 2.43 (95%CI 1.21-4.88; $p=0.013$).

6.5.5 Effect of medication use on gonadal status and male patient survival

Sex hormone and inflammatory mediator levels of patients according to opioid use

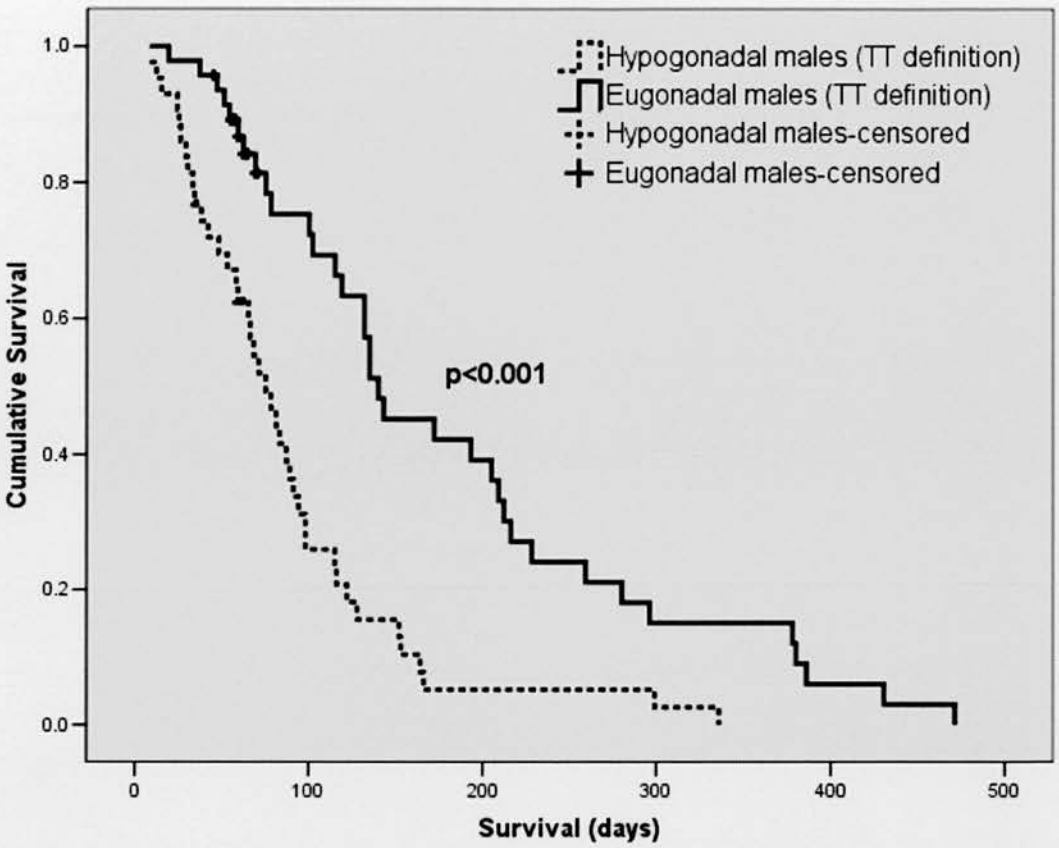

Figure 6.1 **Kaplan-Meier curve of survival of hypogonadal (TT definition) male pancreatic cancer patients.**
Male pancreatic cancer patients with hypogonadism by TT definition demonstrated worsened survival (median 75.0d; 95%CI 57.3-92.8) compared with eugonadal male patients (median 140.0d; 95%CI 95.9-184.1) ($p<0.001$, log rank test).

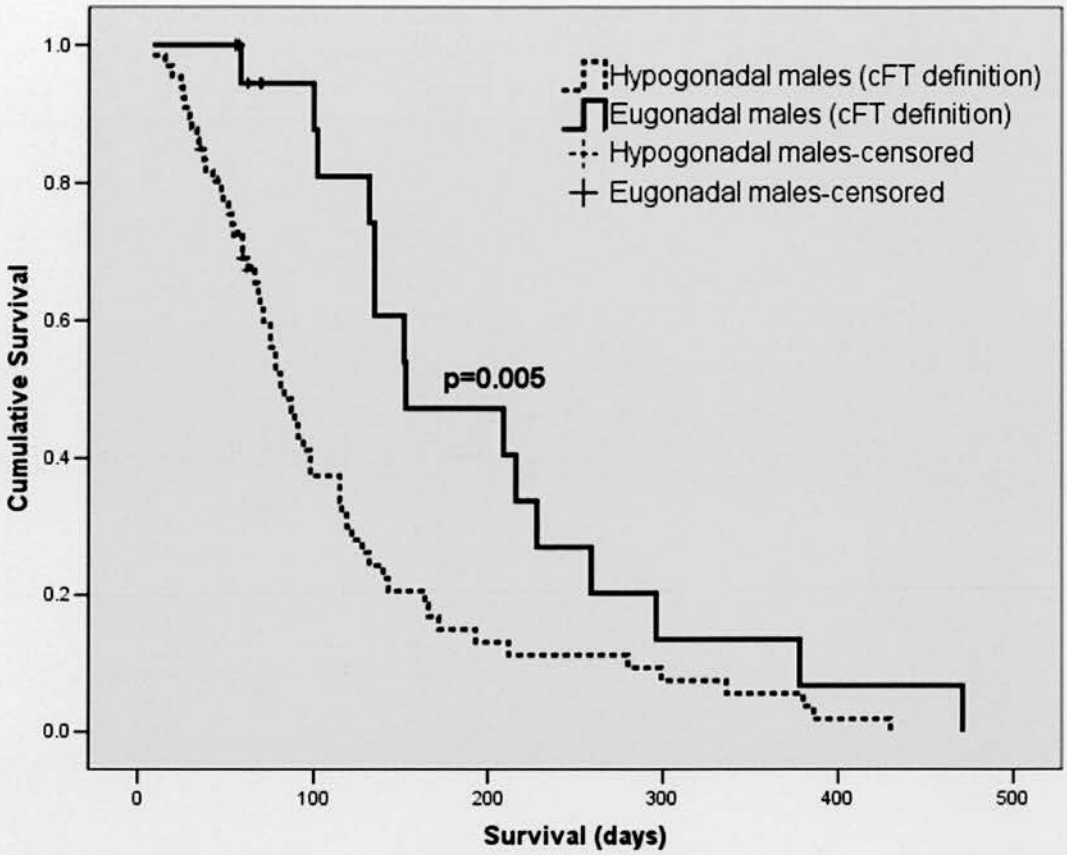

Figure 6.2 **Kaplan-Meier curve of survival of hypogonadal (cFT definition) male pancreatic cancer patients.**
Male pancreatic cancer patients with hypogonadism by cFT definition demonstrated worsened survival (median 83.0d; 95%CI 66.9-99.1) compared with eugonadal male patients (median 153.0d; 95%CI 60.5-245.5) (p=0.005, log rank test).

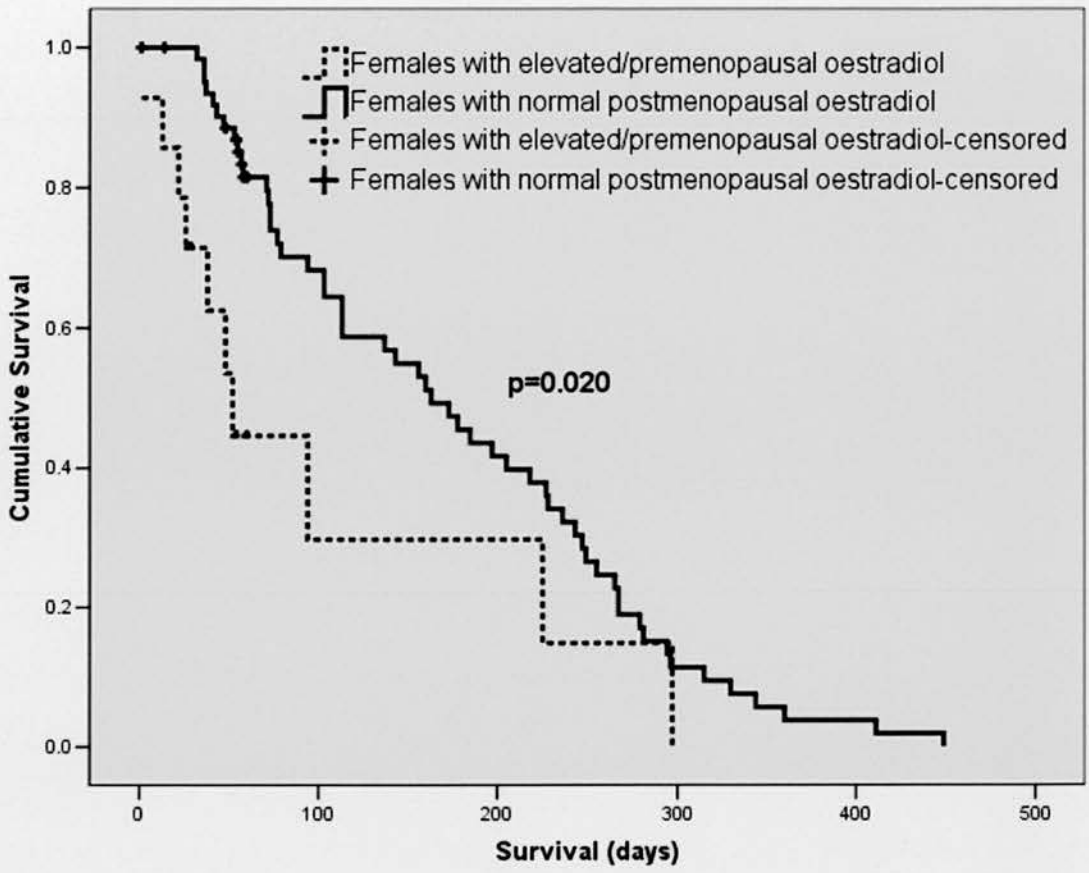

Figure 6.3 **Kaplan-Meier curve of survival of hyperoestrogenal female pancreatic cancer patients.**
Female pancreatic cancer patients with high/premenopausal serum levels of oestradiol (>150pmol/L) demonstrated worsened survival (median 52.0d; 95%CI 29.9 -74.1) compared with female patients who had postmenopausal levels of oestradiol (<150pmol/L) (median 163.0d; 95%CI 114.8-211.2) (p=0.020, log rank test).

are shown in Table 6.3. Twenty-five male (28%) and 18 female (23%) patients used opioids with regular median morphine-equivalence dosages of 80mg (range 0-700) in males and 40mg (range 10-360) in females. Opioid-using males did not differ from non opioid-using males in regard to disease stage or age, whereas opioid-using females exhibited higher disease stage (median stage 4 vs 2; $p=0.009$) and were younger (median age 63yrs vs 70yrs; $p=0.014$). Nutritional variables did not differ between opioid-using and non opioid-using patients of either sex.

In both sexes, opioid use was associated with reduced LH ($p<0.05$) and FSH ($p\leq0.05$), and elevated CRP ($p<0.05$). Furthermore, in males, opioids were associated with a significant reduction in TT ($p<0.001$) and cFT ($p<0.001$). Daily opioid dosage correlated inversely with TT ($r=-0.494$; $p<0.001$), cFT ($r=-0.510$; $p<0.001$) and LH ($r=-0.259$; $p=0.014$), and positively with CRP ($r=0.216$; $p=0.041$) and IL-6 ($r=0.220$; $p=0.037$). On KM analysis, survival was shorter in opioid-using male patients (median 78.0d; 95%CI 59.7-96.3) compared with non-opioid-users (median 132.0d; 95%CI 112.8-151.2) ($p=0.009$) (Figure 6.4). When opioid use, presence of an APPR, percentage weight loss, disease stage and patient age were used as variables in a Cox proportional hazards model, the HR of death associated with opioid use in male patients was 1.96 (95%CI 1.16-3.31; $p=0.012$).

Regarding hormonal medications, insulin and thyroxine use did not affect significantly sex steroid, gonadotropin or pro-inflammatory mediator levels in either sex.

	Male			Female		
	Opioids (n=25)	**No Opioids (n=65)**	***p***	**Opioids (n=18)**	**No Opioids (n=59)**	***p***
Total Testosterone Male 10-30 nmol/L Female 0.4-2.8 nmol/L	5.8 (0.9-18.4)	13.8 (0.3-33.5)	*<0.001*	1.0 (0.5-59.0)	0.9 (0.4-3.1)	*0.957*
Calculated Free Testosterone Male >0.245nmol/L	0.058 (0.005-0.258)	0.187 (0.012-0.568)	*<0.001*	0.009 (0.004-0.864)	0.008 (0.003-0.036)	*0.963*
Oestradiol Male <200 pmol/L Postmenopausal Female <150 pmol/L	N/A	N/A	N/A	76.0 (32.0-1434.0)	75.0 (28.0-2070.0)	*0.759*
Sex hormone-binding globulin Male 6-45 nmol/L Female 30-120 nmol/L	65.4 (11.7-155.0)	65.9 (23.0-335.0)	*0.893*	89.8 (35.0-162.0)	88.3 (39.0-232.0)	*1.000*
Luteinising hormone Male 1.5-9.0 U/L Postmenopausal Female >20 U/L	3.8 (0.3-10.6)	4.7 (1.4-39.7)	*0.019*	0.9 (0.9-4.2)	23.9 (0.9-44.9)	*0.033*
Follicle-stimulating hormone Male 1.5-9.0 U/L Postmenopausal Female >30 U/L	4.6 (0.7-20.1)	7.3 (1.4-55.1)	*0.054*	2.1 (1.0-43.8)	65.6 (1.5-130.2)	*0.030*
CRP Normal <10 mg/L	16.7 (1.0-271.2)	8.9 (0.8-356.9)	*0.039*	25.7 (0.9-337.4)	8.6 (0.9-86.9)	*0.007*
Interleukin-6 Normal 1.3+/-3.2 pg/mL	8.7 (2.0-201.0)	6.5 (1.3-201.0)	*0.109*	7.9 (1.7-127.7)	4.6 (1.5-77.6)	*0.077*
Soluble TNF receptor 75 Normal 5.2+/-2.1 pg/mL	14.2 (6.9-903.0)	12.0 (3.4-994.0)	*0.550*	15.5 (3.7-55.8)	13.2 (6.0-58.9)	*0.416*

Table 6.3 **Serum sex hormone, gonadotropin and pro-inflammatory mediator levels of pancreatic cancer patients according to opioid use.**

Male opioid-using patients demonstrated lower levels of total testsoterone and calculated free testosterone compared with non opioid-using male patients. In both sexes, LH and FSH were lower (or demonstrated a trend towards reduction), whereas CRP was elevated, in opioid-using patients compared with non-opioid-using patients. Data are presented as median values with ranges in parentheses.

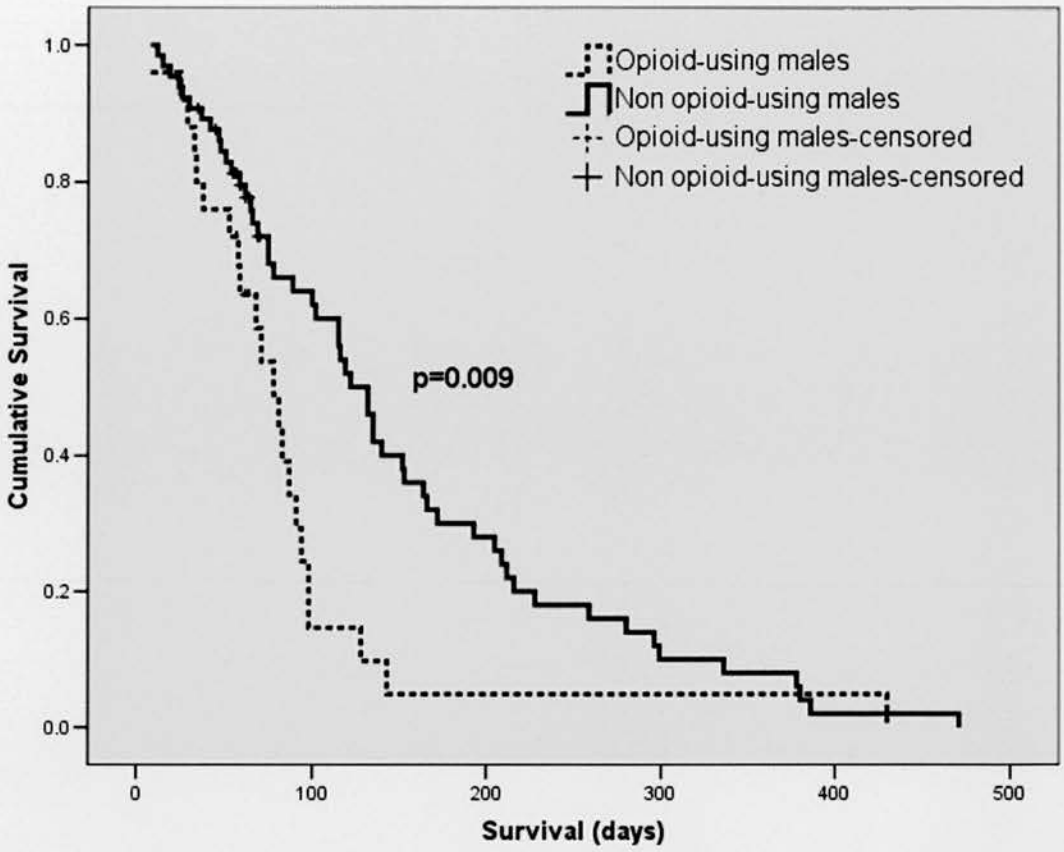

Figure 6.4 **Kaplan-Meier curve of survival of male pancreatic cancer patients according to opioid use.**
Male pancreatic cancer patients using opioids demonstrated worsened survival (median 78.0d; 95%CI 59.7-96.3) compared with male patients who did not (median 132.0d; 95%CI 112.8-151.2) (p=0.009, log rank test).

6.6 Discussion

The present study represents one of the first descriptions of the interaction between hormonal status and systemic inflammation in patients with advanced cancer (non-prostate), and, importantly, the first description of the effects of this interaction on survival. The majority of male patients with unresectable pancreatic cancer were hypogonadal (cFT definition). In contrast, 18% of postmenopausal female patients demonstrated elevated serum oestradiol. Male hypogonadism and female 'hyperoestrogenism' were associated with shortened survival.

Although elevated oestradiol has been documented previously in females with pancreatic cancer [588], the role of androgens in male cancer patients has been studied more widely [101, 178, 586]. Studies of testosterone in this latter group have yielded variable results. Some reports have found both low TT and low cFT [101], whereas others have demonstrated TT similar to controls, and have required measures of cFT and bioavailable testosterone to demonstrate hypogonadism, prompting the conclusion that measures of testosterone bioavailability should always be used [586]. In another study, TT was reduced but cFT was similar to controls [592]. However, many investigations have considered heterogeneous populations of different cancer types [101, 178, 586], whereas the present study has the advantage of a single type.

The present data indicate an interaction between sex steroids and systemic inflammation such that, in males, TT and cFT correlated inversely with inflammatory mediators (particularly CRP and IL-6) whereas, in females, oestradiol

correlated positively with inflammatory mediators (particularly sTNFR75) (Figure 3.5). Furthermore, CRP remained an independent determinant of TT, cFT and oestradiol levels. These interactions appeared to occur outwith the influence of disease stage, as neither circulating levels of inflammatory mediators nor sex steroids correlated with stage. It has been hypothesised that, in men, pro-inflammatory cytokines act directly on the testicles to suppress testosterone [586]. Studies of IL-6 [593] and TNF-α [594] administration to healthy men have demonstrated similar suppression. In postmenopausal women, most oestradiol production is extragonadal. Pro-inflammatory cytokines stimulate aromatase activity in women, thus increasing oestradiol production [590]. Therefore, in the present study, the effects of cytokines are seen independent of ovarian function.

Elevated SHBG has been noted previously in male pancreatic cancer patients [586]. SHBG has an increased affinity for androgens and an elevation in serum level will reduce male cFT, but have minimal impact on female bioavailable oestradiol. It has been hypothesised that SHBG may be a positive acute phase reactant [586]. The present study would support this hypothesis in males as almost half of male patients exhibited an APPR, and SHBG correlated with circulating levels of inflammatory mediators. However, the elevation of SHBG in the presence of systemic inflammation may represent a cancer-specific phenomenon, as neither IL-6 nor TNF-α administered to healthy males affected SHBG levels, even though TT was decreased [593, 594]. In females, SHBG, although elevated, did not correlate with pro-inflammatory mediators. Mechanisms of SHBG elevation in females may

involve indirect effects of altered body mass [595] or a direct effect of oestradiol [590].

Mechanisms by which disturbances in sex steroid levels translate into accelerated demise are not known. Such disturbances might simply represent markers of systemic inflammation [596]. Alternatively, in hypogonadal males, the reduced anabolic stimulus to skeletal muscle by testosterone might drive wasting, thus reducing patient nutritional reserve and performance. The positive association of cFT with both BMI and grip strength in the present study would support this hypothesis. Initially, the observed correlation between BMI and cFT may appear at odds with previous studies that have found an inverse relationship between BMI and testosterone levels. However, many of the previous studies have focused on patients within the overweight or obese BMI ranges [597], whereas the present study features a weight-losing male cohort with a median BMI within the healthy range. The present study is in accordance with other reports that have documented low testosterone levels in anorectic males [598], and high testosterone levels in obese patients with prostate cancer [599]. Thus, hypogonadism could represent an important mechanism of weight loss. However, the iatrogenic induction of hypogonadism in males with prostate cancer only results in loss of LBM at a rate of 2% per 6 months [600]. The short survival duration of pancreatic cancer patients (average 6 months) makes it unlikely that the muscle-wasting effects of hypogonadism alone would be the primary contributor to accelerated demise. Rather, it is likely that hypogonadism is an overall frailty index and that the former interacts with other factors to result in cachexia. These data support the

development of a trial of testosterone treatment in hypogonadal men with advanced pancreatic cancer. However, pancreas is a sex steroid-dependent tissue [601-603] and therefore it is unclear how the primary cancer may respond to supplemental testosterone. Initial studies using flutamide, an anti-androgen, for the treatment of pancreatic cancer were encouraging [604]. However, follow-on studies [605] and trials of gonadotropin-releasing hormone (GnRH) analogues [606] demonstrated no impact on survival. Trials yielding tamoxifen, an oestrogen receptor modulator, either as a single agent therapy or in conjunction with other agents, have also yielded variable results [607-610].

Compared with the proposed mechanisms underlying male hypogonadism and poor prognosis, it is even less clear how elevated oestradiol might adversely affect female nutrition and survival. Post-menopausal loss of oestradiol is considered to be one of the main causes of sarcopenia in women [611]. In the present study, hyperoestrogenal females did not differ from eugonadal females with regard to weight loss, BMI or LBM. Presumably, the interaction between oestradiol and pro-inflammatory mediators remains the key mechanism of worsened survival.

Opioid use is known to induce secondary hypogonadism [587]. Twenty-seven percent of patients in the present study were using opioids, and in males, opioid use was associated with reductions in gonadotropins, TT and cFT in a dose-dependent fashion. Furthermore, opioid use was an independent determinant of testosterone levels and an adverse prognostic factor in male patients, a finding that has perhaps not been fully appreciated in the literature previously [612]. Although LH and FSH

were also reduced in post-menopausal females using opioids, oestradiol synthesis appeared unaffected, presumably reflecting the high extragonadal contribution to oestradiol production. Opioid-using females were younger than their non-using counterparts but the reason why this should be remains unclear.

It is difficult to tease apart cause and effect when considering opioid requirement, uncontrolled pain and patient prognosis. Previous studies have shown pain at diagnosis to be an independent poor prognostic factor in pancreatic cancer [582], suggesting that the host inflammatory response [613], pain-induced stress response or the pattern of tumour invasion might underlie any detrimental response to opioids. In the present study, opioids were associated with an elevated CRP but not necessarily increased disease stage, an observation that could be consistent with either pain-induced stress or an indirect effect of disturbed sex steroids. Thus, the question remains as to whether shortened male survival associated with opioid use is a direct result of the medication or simply a reflection of the fact that opioids are often administered to frailer, hypogonadal patients with stronger systemic inflammatory responses. Previous studies of end-of-life hospice patients have found that increased opioid dosage was associated with reduced time until death, but that this factor explained only very little of the variation in survival [614]. However, the mean survival duration of these hospice patients was only 12 days (compared with 135 days in the present study), and thus study time may have been insufficiently long to assess the inflammatory and muscle wasting effects of opioids over extended time periods. Certainly, the prognostic implications of opioid use would

appear to warrant further investigation, and confirmation of these findings in larger patient groups is needed.

Opioids are clearly of symptomatic benefit to patients with advanced cancer, and so it would be inadvisable to omit them. However, based on the current results, it might be useful to monitor serum gonadotropin levels during opioid use and consider testosterone replacement. Furthermore, non-steroidal anti-inflammatory medications, in combination with androgen replacement therapy, could be used as important co-analgesics with opioid-sparing and life-prolonging effects in hypogonadal males with advanced pancreatic cancer. Clinical trials of such treatment approaches are required.

Limitations of the present study include the fact that gonadal and inflammatory status were assessed on only a single blood test, and that only a small number of controls were recruited. However, it is unlikely that a larger number of controls would have altered the conclusions significantly as median control results lay within the normal range.

In summary, elderly hypogonadal males and "hyperoestrogenic" females with advanced pancreatic cancer appear to be at particular risk of accelerated demise. Although systemic inflammation may contribute to gonadal dysfunction, it remains an independent prognostic indicator. Systemic inflammation in both sexes and hypogonadism in males are likely contributors to the cachexia that affects most patients with progressive disease. Within the context of multimodal supportive care,

this study supports trials of anti-inflammatory/androgen replacement therapy (particularly when combined with opioid analgesia) in males, but a different strategy involving anti-inflammatory therapy in females. Furthermore, this study also supports the use of serum sex steroids as biomarkers of cachexia in pancreatic cancer patients (namely, cFT/TT in males and oestradiol in females), and the use of sex steroid abnormalities as targeting strategies for treatment.

In Part B, possible mediators of muscle wasting and cancer cachexia, including PIF, MIC-1 and sex steroids, have been investigated. Of all of these mediator candidates, only sex steroids have demonstrated robust evidence of a role in cancer-associated wasting. In Part C, rather than human cell lines, tumour samples and patient blood being the tissues of investigation, attention turns to the investigation of skeletal muscle samples from weight-losing cancer patients, in order to elucidate intracellular mechanisms of muscle wasting.

Part C – Mechanisms of Muscle Wasting in Cancer Cachexia

Chapter 7 - Dystrophin glycoprotein complex dysfunction: a regulatory link between muscular dystrophy and cancer cachexia

7.1 Introduction

Part C focuses on the investigation of molecular mechanisms of skeletal muscle wasting in cancer cachexia. In this chapter, the results of an international collaborative study of DGC deregulation in murine and human cancer cachexia are described. This is the first occasion in which DGC deregulation has been investigated in cancer-associated muscle wasting. The results of murine DGC studies are summarised in the Introduction, followed by an analysis of human cachectic muscle samples in the Results section.

In muscle biopsies from cancer patients, wasting is thought to occur from the selective atrophy of type II fibres [321]. Acharyya *et al* demonstrated that muscles from C-26 mice recapitulated faithfully the clinical features of human cancer-induced muscle wasting [321]. Muscles from such mice were severely atrophic, exhibiting 45% reduction in mean fibre diameter. In addition, atrophic fibres were selectively type II in origin. Upon close histological examination, alterations in membrane structure were also apparent. As compared to the smooth and well-defined membranes bordering each myofibre in control muscles, membranes in cachectic murine muscles appeared wrinkled and irregular. This phenotype was confirmed by electron microscopy and laminin staining, giving an early indication that sarcolemma and the associated basal lamina from cachectic muscles were

abnormal. Evans blue dye (EBD) uptake was then used to measure membrane integrity. EBD is normally membrane impermeable, but in damaged myofibres this dye penetrates the sarcolemma and accumulates in the cytoplasm [615]. Tumour-bearing mice injected with EBD revealed visibly higher muscle uptake of the dye compared with control mice. Serum CK can also be used as an indicator of weakened membranes [312]. However, in C-26 mice, CK levels were only elevated moderately over that of controls [321], indicating that although tumour burden causes alterations to muscle membranes or associated basal lamina, the severity of damage is not sufficient to cause a complete permeation of EBD or release of intracellular proteins.

The DGC is a large, membrane-bound, multi-protein structure that forms a strong mechanical link between the cytoskeleton and the ECM, and which plays a major role in regulating membrane integrity [317]. In light of the abnormal membrane morphology seen in the muscles of tumour-bearing mice, it was hypothesised that the DGC might also be involved in the regulation of cancer cachexia. Acharyya *et al* therefore examined the expression and potential regulation of the DGC core member, dystrophin [321]. Western analysis revealed that dystrophin was reduced in muscles from tumour-bearing mice at a time that preceded reductions in mean fibre diameter. In DMD, loss of dystrophin is often associated with compensatory upregulation of its autosomal homologue utrophin [616]. Muscles from tumour-bearing mice also showed similar induction of utrophin. Conditions where dystrophin expression is lost, such as muscular dystrophies or enterovirus-mediated cardiomyopathy, result typically in the concurrent downregulation of other DGC

members [312, 617]. Acharrya *et al* demonstrated that this was not the case in murine cancer cachexia, as expression of other DGC members, including α-DG, β-DG, α-sarcoglycan (α-SG), β-SG, δ-SG and dysferlin, was unaltered [321]. However, detected prominently was the presence of a higher migrating band for both β-DG and β-SG proteins. Aberrant post-translational glycosylation of dystroglycan proteins is known to occur in various disease states [618-620]. Treatment of cachectic muscle lysate with PNGase F not only caused decreases in the MW of the basally glycosylated forms of β-DG and β-SG, but also resulted in almost a complete disappearance of the higher migrating bands, with concomitant increases in endogenous protein [321]. These results suggested that the higher bands represented hyperglycosylated forms of these DGC proteins. No alterations were detected in other glycosylated DGC members (namely, α-SG and δ-SG) or non-DGC glycosylated proteins, such as β1 subunit of ATPase and neural cell adhesion molecule (NCAM). In summary, muscles from C-26 mice demonstrated reduced dystrophin expression, hyperglycosylation of β-DG, and hyperglycosylation of β-SG, a triad of alterations that has been termed 'DGC deregulation'. Subsequent laser capture microdissection (LCM) confirmed a higher intensity of DGC deregulation in cachectic fibres (diameter <30μm) and type II fibres [321]. Furthermore, DGC deregulation was also demonstrated in mice bearing LLC tumours.

To understand the functional relevance of DGC regulation, Acharyya *et al* assessed binding interactions between DGC proteins [321]. In the DGC, α-DG serves as a receptor binding laminin-2 in the ECM [317]. Using ligand overlay assays, a

significant reduction in laminin-α-DG binding was observed in C-26 cachectic muscles. Furthermore, co-immunoprecipitation analysis revealed pronounced decreases in interactions between α-DG and β-DG, and between β-DG and dystrophin.

To establish firmly the contribution of DGC deregulation to murine cancer cachexia, C-26 and LLC tumours were administered to *mdx* mice, which have a mutation that codes for a premature stop codon in the X-linked dystrophin gene [621], resulting in the loss of dystrophin and associated DGC proteins [622]. Although the *mdx* pathology resembles the early phases of DMD, relatively little muscle loss actually occurs in these mice due to the high rate of muscle regeneration and hypertrophy in response to the ongoing cycles of degeneration [312]. Muscle loss was accentuated in tumour-bearing *mdx* mice compared to tumour-bearing wild type littermates [321]. Importantly, fibre membranes from *mdx* mice alone were visibly irregular, and DGC proteins were also glycosylated aberrantly, suggesting that loss of dystrophin leads to β-DG and β-SG alterations. In contrast, transgenic mice expressing a dystrophin minigene in skeletal muscle (Δ17-48) [623] demonstrated little to no decrease in muscle mass or fibre diameter following tumour inoculation. Muscles from Δ17-48 tumour mice were also spared of membrane abnormalities, and showed restored DGC interactions. Furthermore, tumour-bearing wild type mice exhibited a 30% reduction in hindlimb grip strength, while no differences were observed between control and dystrophin transgenic mice [321]. Taken together, these results argued that DGC restoration could both structurally and functionally block tumour-induced muscle changes.

In order to determine whether DGC deregulation also occurs in human cancer cachexia, the present study aimed to examine the expression levels and glycosylation status of DGC members (namely, dystrophin, β-DG and β-SG) in skeletal muscle of patients with OGC and varying degrees of weight loss, in comparison with healthy, weight-stable subjects undergoing minor elective surgery.

7.2 Hypothesis

Skeletal muscle from weight-losing cancer patients demonstrates DGC deregulation (namely, reduction in dystrophin expression, hyperglycosylation of β-DG, and hyperglycosylation of β-SG) compared with weight-stable patients and healthy controls. The presence of DGC deregulation is associated with nutritional depletion, systemic inflammation and worsened survival.

7.3 Patients and healthy controls

Patients with newly diagnosed OGC and varying degrees of weight loss undergoing surgical resection with curative intent (n=27) were recruited (see Methods Chapter 2.1, p.167). Rectus abdominis muscle samples were obtained (see Methods Chapter 2.4.1, p.173) and plasma CRP concentration was measured (see Methods Chapter 2.3.2, p.173). Patients were followed up and underwent sequential nutritional assessment (see Methods Chapter 2.2, p.168). Ten patients (37.0%) died during follow-up. Patients undergoing minor operative procedures for benign, non-inflammatory conditions (n=7) were recruited as controls for comparative analysis (see Methods Chapter 2.1, p.167). Western blots and co-immunoprecipitations for the core DGC members were performed (see Methods Chapter 2.8.1, p.185). A further 7 healthy controls were recruited from the institution performing the molecular biology analyses.

7.4 Statistical analysis

Demographics of the cancer patients and healthy controls are presented as medians with ranges. Differences between groups were determined by MWT or FET. Survival analysis was performed using univariate KM plots.

7.5 Results

The demographics of the weight-losing cancer patients (n=27) and the weight-stable, healthy controls recruited from the author's institution (n=7) are shown in Table 7.1. (A further 7 controls were also recruited for whom nutritional assessment is unavailable). Following post-operative histopathological examination of the tumour specimen, five of the patients had stage 4 disease. Age and sex ratio did not differ significantly between the cancer and control groups. However, cancer patients had lower MAC (p=0.0026) and TSF (p=0.048), and demonstrated a trend towards lower BMI (p=0.059) and MAMC (p=0.054), compared with controls. Moreover, 19/27 (70.4%) of the cancer patients had lost weight prior to resection (median weight loss of all cancer patients = 3.2%; range 0.0-23.2).

Compared to weight-stable healthy controls, weight-losing cancer patients had a dramatic reduction in dystrophin expression (Figure 7.1). Ponceau S staining confirmed that loss of dystrophin was not due to a general reduction of total protein (Figure 7.1). Similar to the changes observed in murine models of cancer cachexia, in carcinoma patients, dystrophin downregulation was tightly linked with hyperglycosylation of β-DG and β-SG, to the extent that a direct correlation could be distinguished clearly between dystrophin reduction and increased β-DG and β-SG glycosylation (Figure 7.1). In total, DGC deregulation was found in 17/27 (63.0%) of cancer samples, but was completely absent from controls (n=14). Moreover, analogous to the C-26 murine cachexia model, ligand overlay assays with muscle extracts from cancer patients revealed decreases in DGC complex association (Figure 7.2A). Results also highlighted that wasting was not selective to

	Healthy Controls (Weight-Stable)	Cancer Patients
Number (n)	7	27
Sex		
Male	6	20
Female	1	7
Age (yrs)	53 (24-85)	65 (45-83)
Tumour Site		
Oesophageal	N/A	11
Gastro-oesophageal junction		4
Gastric		12
Histology		
Adenocarcinoma	N/A	27
Squamous		0
Stage		
I	N/A	7
II		7
III		8
IV		5
Body Mass Index (kg/m^2)	29.0 (19.6-35.2)	25.5 (17.8-40.6)
Mid-Arm Circumference (cm)	32.6 (25.5-36.2)	27.5[b] (22.0-36.0)
Triceps Skinfold Thickness (mm)	16.0 (5.4-35.4)	11.0[a] (5.0-31.0)
Mid-Arm Muscle Circumference (cm)	27.5 (23.8-30.5)	24.2 (18.2-28.5)
CRP (mg/L)	6 (1-20)	7 (1-98)
Karnofsky Performance Score	100 (100-100)	100 (70-100)
% Weight Loss (% loss of pre-morbid weight)	0 (0.0-0.0)	3.2[b] (0.0-23.2)

Table 7.1 **Demographics of the weight-losing oesophago-gastric cancer patients and weight-stable, healthy controls.**
Mid-arm circumference and triceps skinfold thickness were significantly lower in cancer patients compared with controls, whereas body mass index and mid-arm muscle circumference demonstrated a trend towards being reduced. Cancer patients exhibited a median weight loss of 3.2%. Data are presented as medians with ranges in parentheses. Differences from healthy controls are shown as as a, $p<0.05$; b, $p<0.01$.

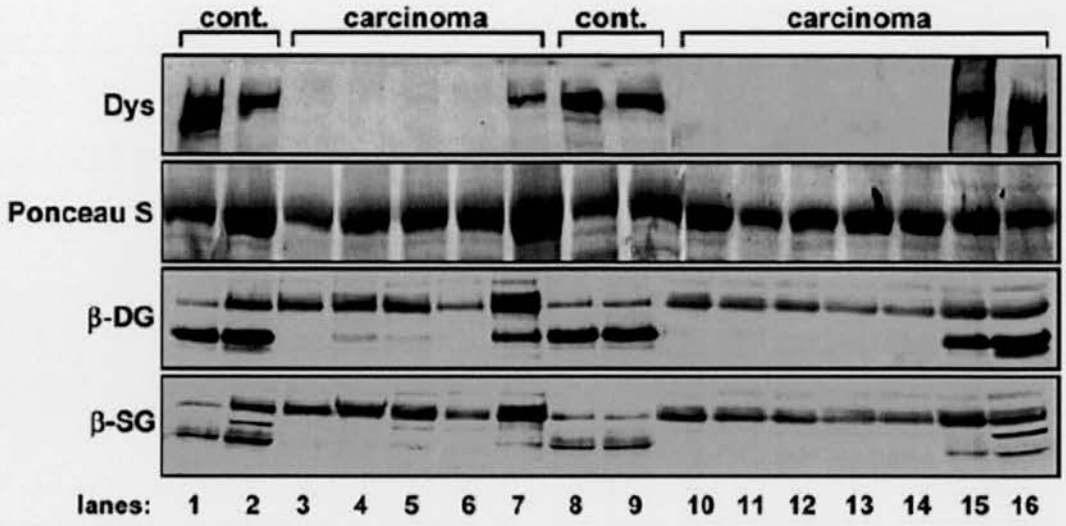

Figure 7.1 **Dystrophin glycoprotein complex deregulation in rectus abdominis samples from weight-losing cancer patients.**
Rectus abdominis biopsies from controls (cont.) and cancer patients were homogenised and analysed by Western blots probing for dystrophin (Dys), β-dystroglycan (β-DG), and β-sarcoglycan (β-SG). Upper bands denote hyperglycosylated products. Muscle samples from cancer patients demonstrated reduced dystrophin expression, hyperglycosylated β-DG, and hyperglycosylated β-SG.

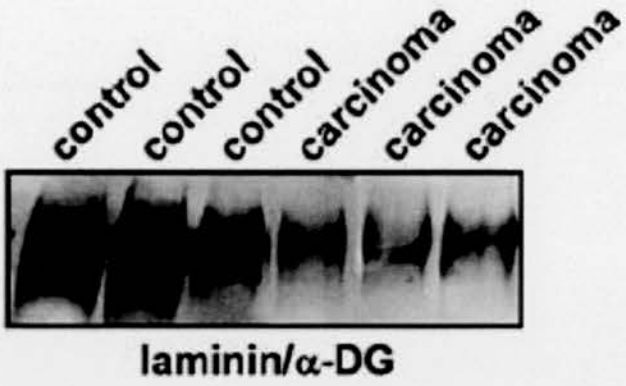

Figure 7.2 A

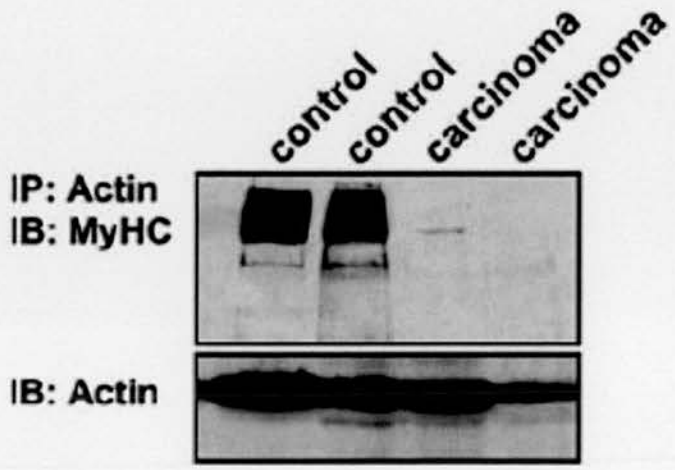

Figure 7.2 B

Figure 7.2 **A) Ligand overlay assays performed with muscles from cancer patients and controls.**
Weight-losing cancer patients demonstrated decreases in DGC complex association.
B) Co-immunoprecipitations performed with samples from 7.2A with a skeletal muscle actin antibody and immunoblotted subsequently for myosin heavy chain.
The wasting phenomenon was not specific to the DGC as cancer patients also demonstrated decreases in actin-myosin complex association. Corresponding actin Western is also shown.
IB: immunoblot; IP: immunoprecipitation; MyHC: myosin heavy chain.

the DGC, since similar deregulation occurred in actin-myosin complexes (Figure 7.2B).

The demographics of the cancer patients with deregulated DGC (n=17) and patients with normal DGC (n=10) are shown in Table 7.2. Patients expressing deregulated DGC demonstrated higher plasma levels of CRP (p=0.0046, MWT) compared with patients with normal DGC, and demonstrated a trend towards lower KPS (p=0.052, MWT). Furthermore, weight loss was increased in patients with deregulated DGC compared with patients with normal DGC (median 6.6% vs 0.2%), although this result did not reach statistical significance (p=0.14).

In order to investigate if deregulated DGC might be used as an early marker of cachexia and therefore capable of predicting the future development of cachexia and overall survival, data were analysed with regards to sequential nutritional assessments. When cachexia was defined strictly as the simultaneous presence of weight loss $\geq$10% and CRP $\geq$10mg/L [624], 10/11 (91%) patients who progressed to demonstrate these findings exhibited prominent DGC deregulation (p=0.018, FET). Furthermore, survival analysis demonstrated that all 10 (100%) non-surviving cases were positive for DGC deregulation. Furthermore, KM analysis demonstrated that patients with deregulated DGC demonstrated significantly shortened survival (median 479d) compared with patients with normal DGC (p=0.013, log rank test) (Figure 7.3).

	Cancer Patients Normal DGC	Cancer Patients Deregulated DGC
Number (n)	10	17
Sex		
Male	7	13
Female	3	4
Age (yrs)	53 (45-83)	64 (48-80)
Tumour Site		
Oesophageal	5	6
Gastro-oesophageal junction	2	2
Gastric	3	9
Histology		
Adenocarcinoma	10	17
Squamous	0	0
Stage		
I	3	4
II	4	3
III	2	6
IV	1	4
Body Mass Index (kg/m^2)	24.8 (19.5-28.7)	25.5 (17.8-40.6)
Mid-Arm Circumference (cm)	27.3 (22.0-32.0)	28.0 (22.5-36.0)
Triceps Skinfold Thickness (mm)	11.3 (8.5-14.0)	11.0 (5.0-31.0)
Mid-Arm Muscle Circumference (cm)	24.1 (18.2-29.2)	24.4 (19.9-28.5)
CRP (mg/L)	1 (1-24)	10[a] (1-98)
Karnofsky Performance Score	100 (80-100)	90 (70-100)
% Weight Loss (% loss of pre-morbid weight)	0.2 (0.0-13.9)	6.6 (0.0-23.2)

Table 7.2 **Demographics of the cancer patients with deregulated DGC compared with patients with normal DGC.**
Plasma CRP was significantly elevated in cancer patients with deregulated DGC compared with patients with normal DGC. Data are presented as medians with ranges in parentheses. Differences from cancer patients with normal DGC are shown as as a, $p<0.01$.

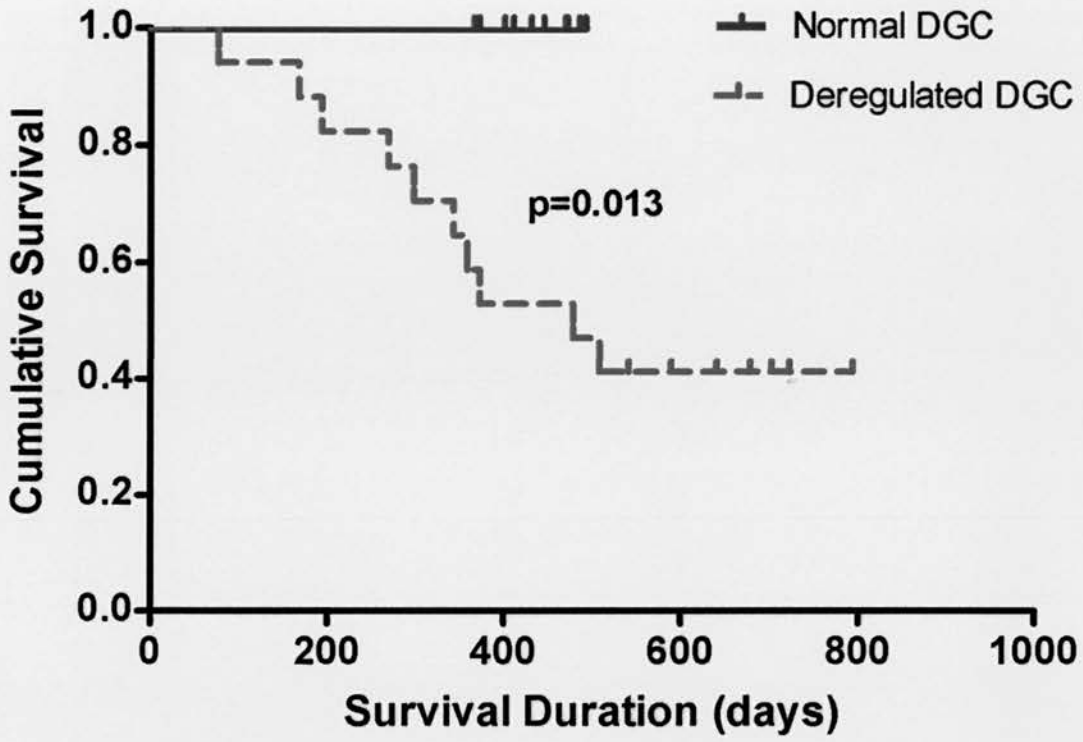

Figure 7.3 **Kaplan-Meier curve of survival of oesophago-gastric cancer patients according to the presence of DGC deregulation within skeletal muscle.** All patients who died during follow-up (n=10) exhibited DGC deregulation. Furthermore, patients with DGC deregulation (median survival 479d) demonstrated worsened survival compared with patients with normal DGC (p=0.013, log rank test).
DGC: dystrophin glycoprotein complex.

7.6 Discussion

Although the underlying mechanisms of cancer cachexia are considered distinct from those seen in muscular dystrophy, the present findings suggest that a dysfunctional DGC might be a common link between these two disease states. This conclusion was based initially on histological and ultrastructural analysis of muscle from tumour-bearing mice, which revealed distinct morphological changes in myofibre membranes [321]. These changes were associated with a reduction in dystrophin expression and the appearance of glycosylated modifications on selective members of the DGC. Tumour-induced alterations in the DGC also correlated with reduced protein interactions within the complex. Indeed, muscle wasting was exacerbated in tumour-bearing *mdx* mice that lacked dystrophin and a functional DGC, but this wasting was abrogated in dystrophin transgenic mice bearing similar tumour burden. Lastly, DGC deregulation (namely, reduced expression of dystrophin, hyperglycosylation of β-DG and hyperglycosylation of β-SG) was identified in 63% of weight-losing cancer patients, and was associated with worsened survival on univariate analysis.

Although cancer cachexia and muscular dystrophy involve chronic muscle wasting, from an aetiological and molecular standpoint, the causative mechanisms of these disorders have been considered to be largely non-overlapping [320, 625, 626]. This is because muscular dystrophies are genetic in origin, with numerous mutations mapping to dystrophin and other members of the DGC. Moreover, features of dystrophy include the recruitment of infiltrating T-lymphocytes and macrophages; necrosis; muscle regeneration; apoptosis; and accumulation of fibrotic material and

fatty deposits, that together culminate in a degenerative state [311, 317]. Biopsies from cancer patients [627], and the C-26 murine model revealed little if any evidence of infiltrating cells, necrosis, regeneration, or fibrosis [321]. Rather, muscle loss due to malignancy exhibited pronounced fibre atrophy and increased vascularisation, which may serve to facilitate circulation of tumour factors and host cytokines in the muscle microenvironment. Despite the differences between cachexia and muscular dystrophy, the present findings indicate that one important commonality is the abnormality of fibre membranes resulting from a deregulated DGC. Interestingly, disruptions and irregularities of the membrane have also been reported in DMD patients as secondary consequences of the loss of the DGC [628, 629]. In muscular dystrophy, mutations in DGC proteins are thought to weaken muscle membranes, which can be measured by EBD permeability or the efflux of cytoplasmic muscle proteins such as CK [630]. In muscles from mice bearing C-26 tumours, enhanced EBD association to membranes was clearly visible, but only on rare occasions was cytoplasmic localisation of the dye observed, and significant CK release was not noted [321]. This would indicate that alterations in fibre membranes occur in cachectic fibres, but to a lesser degree than in dystrophic muscles. It is speculated that this difference in membrane damage correlates with the degree of dysfunction exhibited by the DGC. For example, in DMD, the loss in dystrophin leads to a reduction of DG and SG proteins, resulting in the functional breakdown of the DGC [631]. In the human cancer patients and the C-26 model of cancer cachexia, dystrophin expression may be reduced, but the loss of other DGC proteins was not observed [321]. Nevertheless, evidence from the *mdx* and dystrophin transgenic mice indicates that the alterations in the DGC elicited by a developing

tumour are sufficient to induce membrane damage that may function as a cause and not simply a consequence of muscle wasting.

In conjunction with the downregulation of dystrophin and a compensatory response in utrophin expression, two other key members of the DGC, β-DG and β-SG, were glycosylated aberrantly in mouse tumour models and cachectic patients [321]. The extent of this glycosylation appeared to be linked closely with the level of dystrophin reduction. β-DG and β-SG glycosylation occurred in multiple mouse muscles and by LCM was found to be specific to atrophic type II fibres. The observation that these glycosylated forms appear in *mdx* muscle lends genetic support to the supposition that loss of dystrophin expression leads to aberrant glycosylation in DGC proteins. Deregulation of these DGC proteins correlated with a strong loss of binding between β-DG and dystrophin, α-DG and β-DG, and α-DG and laminin [321]. It is hypothesised that the decrease in dystrophin contributes to the reduction in these interactions, but further studies are warranted to determine the significance of the increase in glycosylation pattern with respect to the breakdown of the DGC and promotion of muscle wasting. In certain congenital dystrophies, mutations arise in glycosyltransferases, which decrease rather than increase glycosylation of DGC proteins, such as α-DG [619, 620]. However, overexpression of a glycosyltransferase specifically in muscle has also been shown to lead to a decrease in fibre diameter [632]. Thus, it is possible that increases in glycosyltransferase activity in tumour-bearing mice may be a contributing factor in cachexia.

It is not known whether the tumour-induced deregulation of the DGC leads to muscle wasting via a breakdown in the mechanical link between the ECM and the cytoskeletal network that interacts with the contractile proteins, or through a disruption in the signalling link between the DGC and MAPK or the PI3K/Akt pathways [633]. Data showing that β-DG/Grb2 interaction, as well as extracellular signal-regulated kinase (ERK) and Akt activities, is maintained in cachectic muscles would support the hypothesised connection between a disrupted mechanical link and muscle wasting. However, it is equally likely that tumour-induced alterations in the DGC may abrogate a separate, yet to be identified pathway that is critical to the promotion of wasting. Such a pathway could be the UPP. The accentuated reduction of MyHC in tumour-bearing *mdx* mice, and the blockage of muscle wasting and E3 ubiquitin ligase (MuRF1 and atrogin-1/MAFbx) induction that were observed in dystrophin transgenic mice [321], both argue for a role of deregulated DGC in proteasome regulation. Taken together, the results support a model that during tumour progression circulating tumour factors function to downregulate dystrophin expression leading to aberrant glycosylation of β-DG and β-SG proteins. These regulatory events subsequently promote DGC breakdown followed by membrane abnormalities. A dysfunctional DGC, either alone or in combination with other deregulated factors associated with an altered membrane or basal lamina, in turn stimulates the induction of E3 ligase genes and possibly other components of the UPP required for protein degradation, culminating in a cachectic state.

In weight-losing cancer patients, DGC deregulation was associated significantly

with a higher plasma level of CRP, suggesting that DGC deregulation may be the result of systemic inflammation. This finding in humans would be consistent with the observations from murine studies, as the C-26 cachexia model is purported to be IL-6-dependent. Certainly, within the present collaborative study, C-26 mice did exhibit higher serum levels of IL-6 [321]. DGC deregulation in cancer patients was also associated with a greater degree of weight loss, although this result did not reach statistical significance. Although these studies suggest heavily that DGC deregulation is potentially a key mechanism of muscle wasting in human cancer cachexia, future studies of larger patient numbers or specific assessments of skeletal muscle mass/LBM may be required to prove this conclusively and avoid the potential risk of type I errors. Moreover, in studies of larger patient numbers, receiver operating characteristic (ROC) curves could be utilised to assess the relationship between DGC deregulation and different clinical definitions of cachexia. The observation that reduced dystrophin expression was seen prior to decreases in muscle fibre diameter in tumour-bearing mice implies that DGC deregulation may hold promise as an early marker of cancer cachexia.

Interestingly, in the present study, DGC deregulation also demonstrated a trend towards association with lower patient KPS, suggesting that DGC deregulation may not only represent a potential marker of muscle wasting, but also a marker of impaired muscle function. This concept is supported by the maintenance of muscle function observed in dystrophin transgenic mice bearing tumours. The notion of an invasive biomarker of muscle function is an intriguing one, which could be combined with other clinical biomarkers (e.g. PA – see Chapter 10, p.339, and

Chapter 11, p.359) to obtain a global picture of patient PF.

Importantly, DGC deregulation was associated with shortened survival of cancer patients on univariate analysis. Thus, despite the fact that all patients within the study were planned for surgical resection with "curative intent", DGC deregulation was capable of predicting survival before the implementation of surgery. Again, larger patient numbers would allow multivariate survival analyses to ascertain the independent prognostic significance of DGC deregulation, and the ability of DGC dergulation to function as a marker of survival.

Collectively, evidence presented in this chapter suggests that DGC dysfunction is an important step in the initiation of cancer cachexia in both murine cancer models and human cancer patients. Since effective therapies are currently lacking, results from this study also imply that approaches targeted to restore the DGC in muscular dystrophies could also be considered as a viable option in designing anti-cancer cachexia therapies. In Chapter 8, attention remains on intra-muscular mechanisms of wasting, and focuses more specifically on molecular pathways involved in protein synthesis and degradation.

Chapter 8 - Increased expression of phosphorylated forms of RNA-dependent protein kinase and eukaryotic initiation factor 2α may signal skeletal muscle wasting in weight-losing cancer patients

8.1 Introduction

Skeletal muscle atrophy is characterised by decreased protein content, fibre diameter, force production and fatigue resistance. Muscle wasting is due to a combination of depressed protein synthesis [634], and elevated endogenous protein breakdown, with oxidation of the resultant amino acids [635]. In cancer patients, the mechanism for the depression in protein synthesis is not known, while the increased protein degradation has been attributed to an increased expression of the UPP [360].

Several potential mediators of the cachectic process, including murine PIF and angiotensin II, have been proposed to inhibit protein synthesis in murine skeletal muscle [170, 545], and also stimulate protein degradation, through increased activity and expression of the UPP [169, 185]. In murine myotubes [188], a link between the ability of murine PIF and angiotensin II to inhibit protein synthesis and increase protein degradation via PKR has been demonstrated. PKR is a serine/threonine specific protein kinase, which undergoes autophosphorylation at multiple serine and threonine residues, causing activation, in the presence of interferon, in response to viral attack [636]. In pre-clinical models, both murine PIF and angiotensin II were shown to induce autophosphorylation of PKR. Activated

PKR can phosphorylate several protein substrates including the α-subunit of eIF2 [637]. The eIF2 complex initiates methionyl-transfer RNA (tRNA) binding to the 40S ribosomal subunit. However, phosphorylation of eIF2α inhibits continued initiation of protein synthesis by this complex. During this process, GTP associated with eIF2 is hydrolysed to guanosine diphosphate (GDP), and recycling of eIF2-GDP to eIF2-GTP requires a guanine nucleotide exchange factor, eIF2B [638]. Phosphorylation of eIF2 on the α-subunit causes it to act as an inhibitor of eIF2B, and the resultant reduction in eIF2-GTP levels reduces general translation (Figure 8.1) [639]. Activation of PKR by murine PIF and angiotensin II was responsible for the observed depression in protein synthesis, since transfection of myotubes with a mutant PKR incapable of autophosphorylation and induction of phosphorylation of eIF2α attenuated completely the depression in protein synthesis by both agents [188]. Mutation of PKR also attenuated completely the induction of protein degradation and upregulation of the UPP. Induction of the UPP by both murine PIF [640] and angiotensin II [174] requires activation of NF-κB. PKR has been shown to activate upstream IKK, leading to degradation of the inhibitor IκB protein and the release of NF-κB, which migrates to the nucleus and induces transcriptional activation of specific genes [641], leading to activation of the UPP (Figure 8.1). Thus, activation of PKR leads potentially to both a depression of protein synthesis and an increase in protein degradation in skeletal muscle. These studies *in vitro* were also reflected by changes *in vivo* in gastrocnemius muscle of mice bearing a cachexia-inducing tumour, where levels of phosphorylated PKR and eIF2α were found to rise with increasing weight loss (by as much as 18-fold for PKR at 25% weight loss) [188].

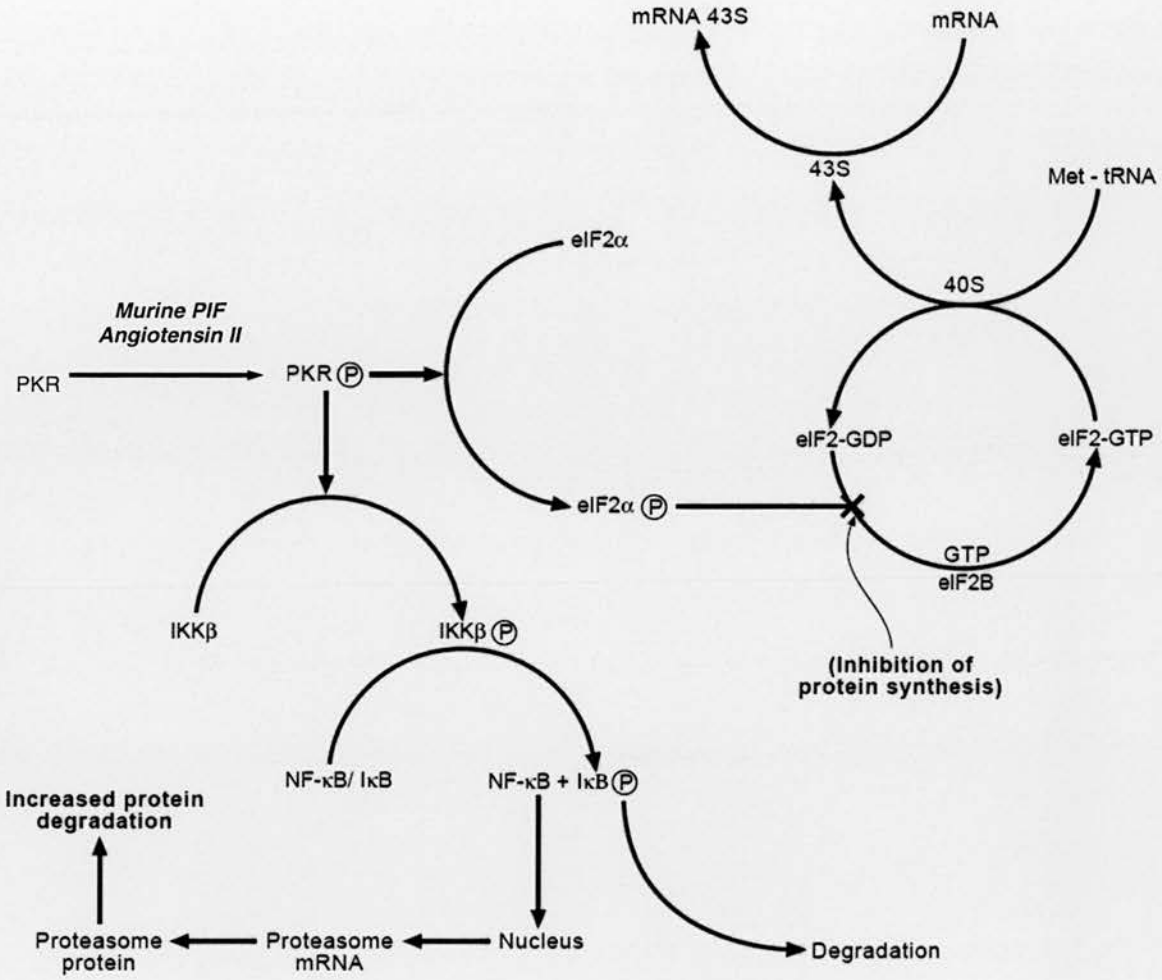

Figure 8.1 **Summary of pathways leading to a depression in protein synthesis and an increase in protein degradation in skeletal muscle through phosphorylation of PKR.**

During skeletal muscle wasting, phosphorylation (denoted by a P in a circle) of PKR not only leads to phosphorylation of eIF2α and an inhibition of protein synthesis, but also to phosphorylation of IKK, leading to NF-κB-mediated protein degradation. *In vitro*, phosphorylation of PKR has been induced by murine PIF and angiotensin II.

eIF2α = eukaryotic initiation factor 2α; GDP = guanosine diphosphate; GTP = guanosine triphosphate; IKK = IκB kinase; Met-tRNA = methionyl transfer-RNA; NF-κB = nuclear factor-κβ; PKR = double-stranded RNA-dependent protein kinase.

In order to determine whether changes similar to those induced by murine PIF and angiotensin II *in vitro* also occur in human cancer cachexia, the present study aimed to examine the expression levels of phosphorylated PKR and eIF2α in skeletal muscle of patients with OGC and varying degrees of weight loss, in comparison with healthy, weight-stable subjects undergoing minor elective surgery.

8.2 Hypothesis

Skeletal muscle from weight-losing cancer patients demonstrates increased levels of phosphorylated PKR and phosphorylated eIF2α expression compared with weight-stable patients and healthy controls.

8.3 Patients and healthy controls

Patients with newly diagnosed OGC and varying degrees of weight loss undergoing surgical resection with curative intent (n=14) or without curative intent (n=1) were recruited (see Methods Chapter 2.1, p.167) and rectus abdominis muscle samples were obtained (see Methods Chapter 2.4.1, p.173). Nutritional assessment was performed (see Methods Chapter 2.2, p.168). Patients undergoing minor operative procedures for benign, non-inflammatory conditions (n=9) were recruited as controls for comparative analysis (see Methods Chapter 2.1, p.167). Western blots for the phospho and total forms of PKR and eIF2α, MyHC and actin in skeletal muscle were performed in triplicate (see Methods Chapter 2.8.2, p.186).

8.4 Statistical analysis

Demographics of the cancer patients and healthy controls are presented as medians with ranges. Differences between the two groups were determined by MWT. Western blot densitometry results are presented as means ± standard error of the mean (SEM) for at least three replicate experiments. Differences in means between groups were determined by one-way ANOVA, followed by the Tukey-Kramer multiple comparison test. Correlation analysis was performed using Spearman's Rank Correlation Coefficient.

8.5 Results

The demographics of the weight-losing cancer patients (n=15) and the weight-stable, healthy controls (n=9) are shown in Table 8.1. Following post-operative histopathological examination of the tumour specimen, three of the patients had stage 4 disease. Age and sex ratio did not differ significantly between the cancer and control groups. Thirteen (86.6%) of the cancer patients had lost weight prior to resection (median weight loss of all cancer patients = 7.8%; range 0.0-27.5). However, no cancer patient had a BMI less than 20 kg/m^2, and median BMI of the patients was not significantly lower than the control group (26.1 kg/m^2 vs 28.5 kg/m^2; p=0.311).

Western blots for the phospho and total forms of PKR and eIF2α in rectus abdominus muscle as a function of weight loss are shown in Figures 8.2 and 8.3. Levels of phospho PKR were elevated significantly compared with healthy controls in 13 (86.6%; $p<0.05$) of the muscle samples from cancer patients, whereas phospho eIF2α levels were enhanced significantly in 9 (60.0%; $p<0.05$). There did not appear to be a significant relationship between the degree of patient weight loss and the level of mediator phosphorylation. Moreover, one weight-stable cancer patient demonstrated significantly elevated levels of intramuscular phospho PKR ($p<0.01$). However, there was a linear correlation between phosphorylation of PKR and phosphorylation of eIF2α ($r=0.76$, $p=0.005$), suggesting that phosphorylation of PKR was associated with, and may have led to, phosphorylation of eIF2α (Figure 8.4). Muscle myosin (MyHC II) levels decreased significantly as patient weight loss increased beyond 5% (Figure 8.5A), whereas actin levels remained stable.

	Healthy Controls (Weight-Stable)	Cancer Patients
Number (n)	9	15
Sex		
Male	9	13
Female	0	2
Age (yrs)	56 (41-86)	66 (49-83)
Tumour Site		
Oesophageal	N/A	6
Gastric		8
Histology		
Adenocarcinoma	N/A	14
Squamous		1
Stage		
I	N/A	3
II		3
III		6
IV		3
Body Mass Index (kg/m^2)	28.5 (19.6-35.2)	26.1 (20.1-34.4)
Mid-Arm Circumference (cm)	32.6 (25.5-35.2)	28.9 (23.0-40.0)
Triceps Skinfold Thickness (mm)	15.5 (5.0-29.4)	14.4 (7.8-37.4)
Mid-Arm Muscle Circumference (cm)	25.8 (23.8-30.5)	25.3 (18.3-30.0)
Karnofsky Performance Score	100 (100-100)	90[a] (60-100)
% Weight Loss (% loss of pre-morbid weight)	0	7.8[b] (0.0-27.5)

Table 8.1 **Demographics of the weight-losing oesophago-gastric cancer patients and weight-stable, healthy controls.**
Although median BMI was not significantly different between cancer patients and controls, the cancer patients did exhibit a median weight loss of 7.8%. Data are presented as medians with ranges in parentheses. Differences from healthy controls are shown as as a, $p<0.05$; b, $p<0.001$.

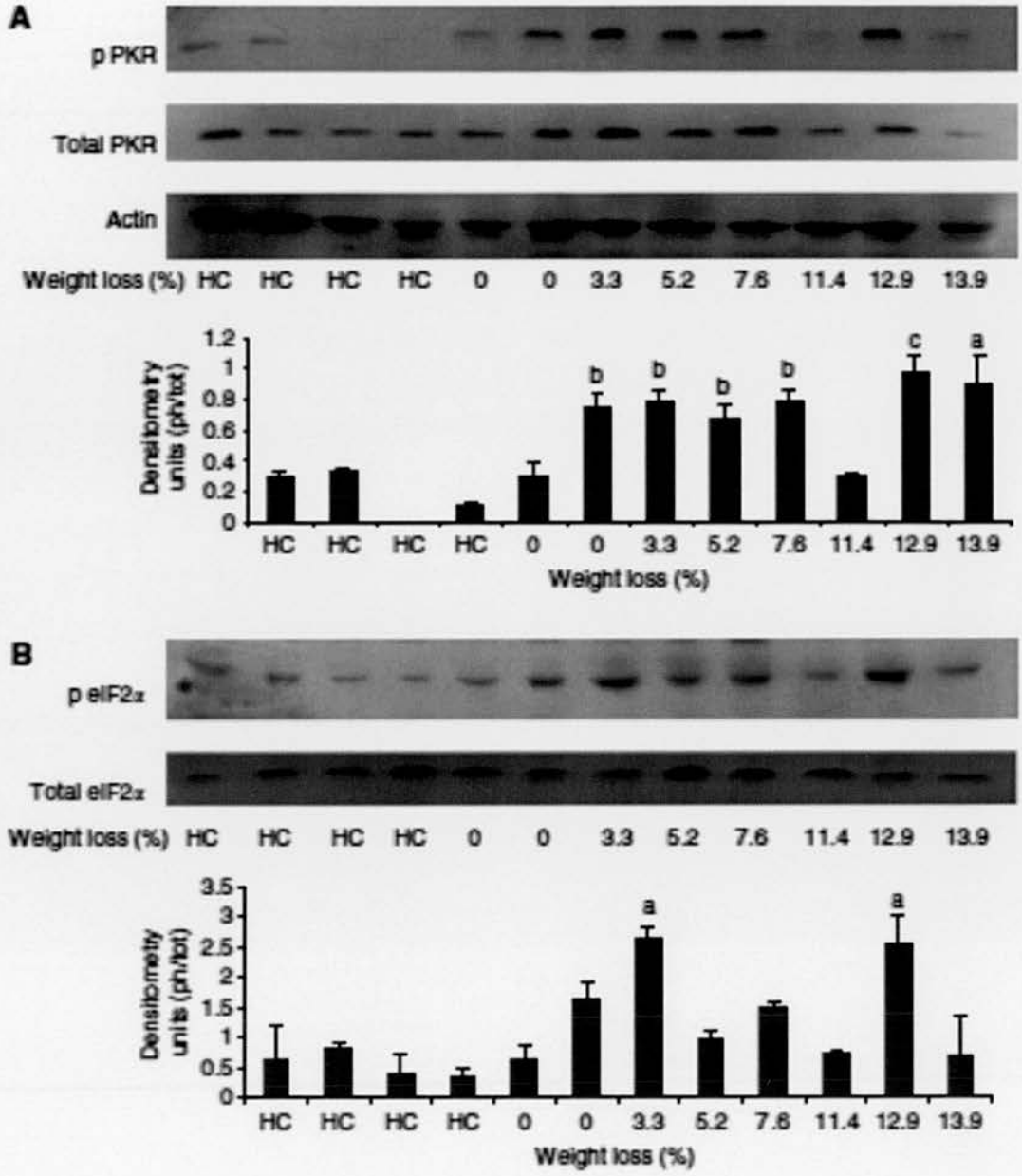

Figure 8.2 **Western blots of phospho PKR (A) and eIF2α (B) compared with total PKR and eIF2α in rectus abdominus muscle as a function of weight loss.**

The ratio of phospho to total PKR and eIF2α was greater in weight-losing cancer patients. Actin was used as a loading control. Each lane represents muscle from an individual patient. Densitometric analysis of the ratio of phospho to total forms represents the average of 3 separate blots. Differences from healthy controls are shown as as a, $p<0.05$, b, $p<0.01$ or c, $p<0.001$.

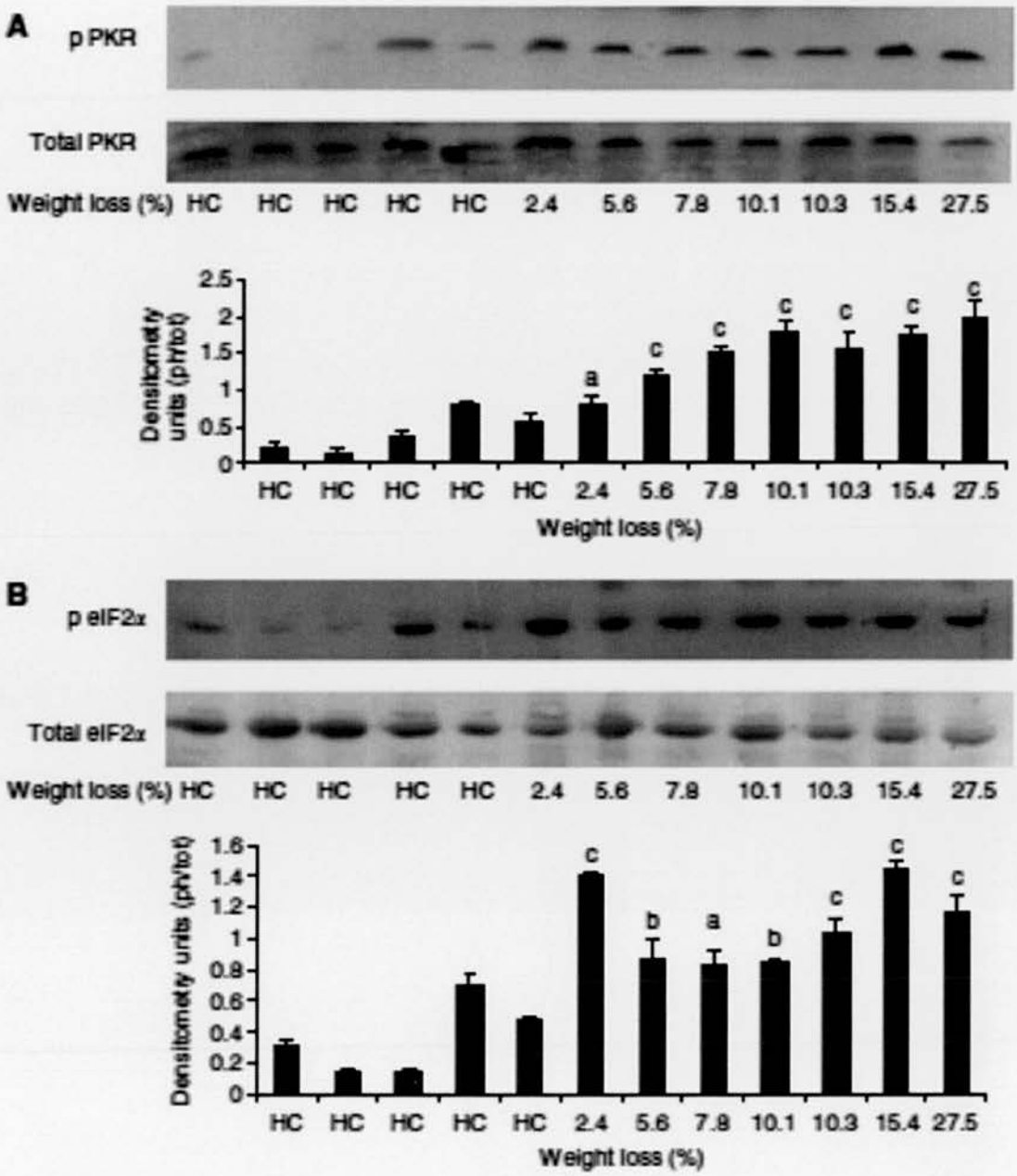

Figure 8.3 **Western blots of phospho PKR (A) and eIF2α (B) compared with total PKR and eIF2α in rectus abdominus muscle as a function of weight loss.** The ratio of phospho to total PKR and eIF2α was greater in weight-losing cancer patients. Actin was used as a loading control. Each lane represents muscle from an individual patient. Densitometric analysis of the ratio of phospho to total forms represents the average of 3 separate blots. Differences from healthy controls are shown as as a, $p<0.05$, b, $p<0.01$ or c, $p<0.001$.

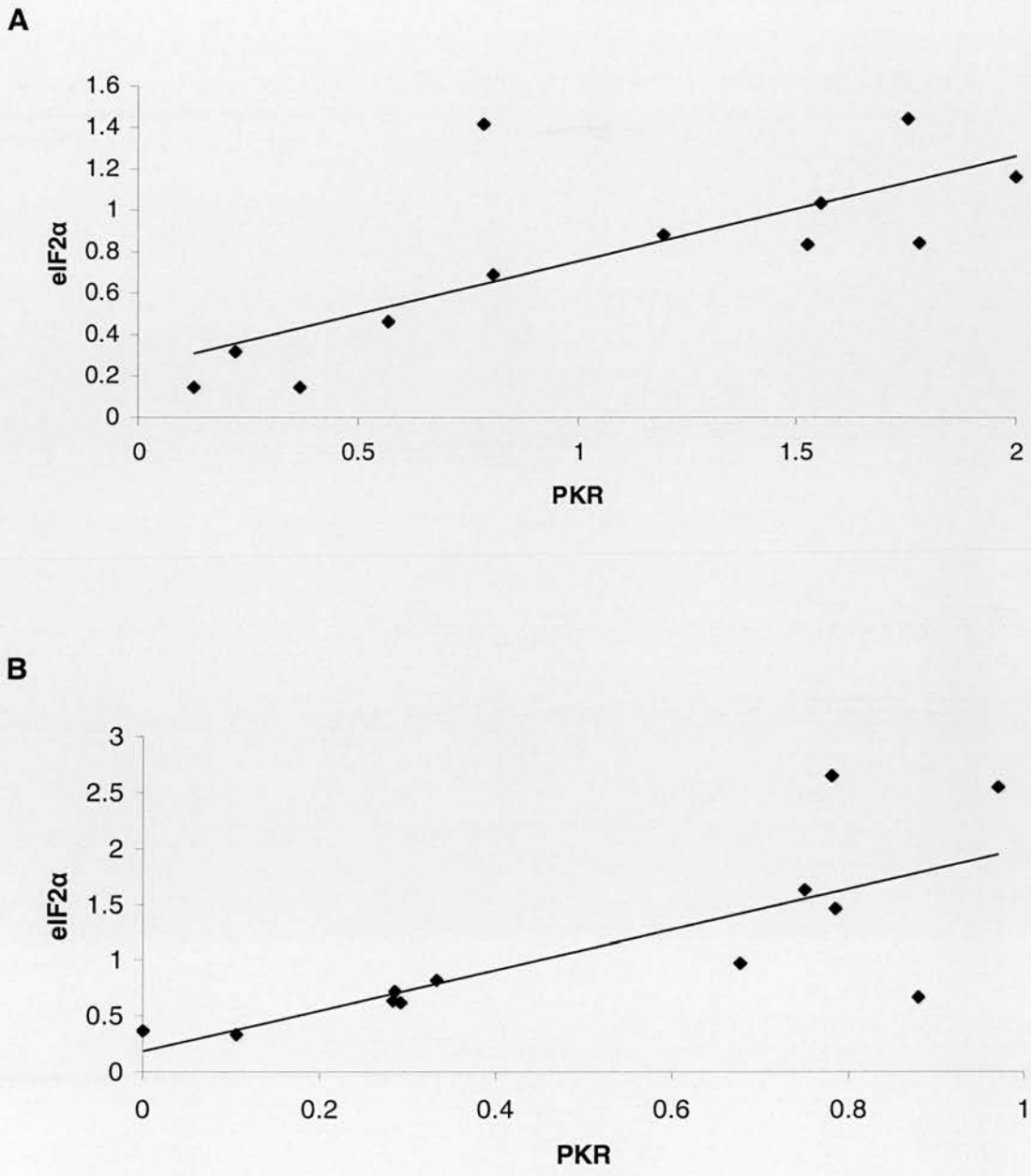

Figure 8.4 **Relationship between the level of autophosphorylation of PKR and eIF2α in skeletal muscle samples from: A) patients shown in Figure 8.2; and B) patients shown in Figure 8.3.**
There was a positive linear correlation between phosphorylation of PKR and phosphorylation of eIF2α (r=0.76, p=0.005 for both graphs), suggesting that phosphorylation of PKR was associated with phosphorylation of eIF2α.

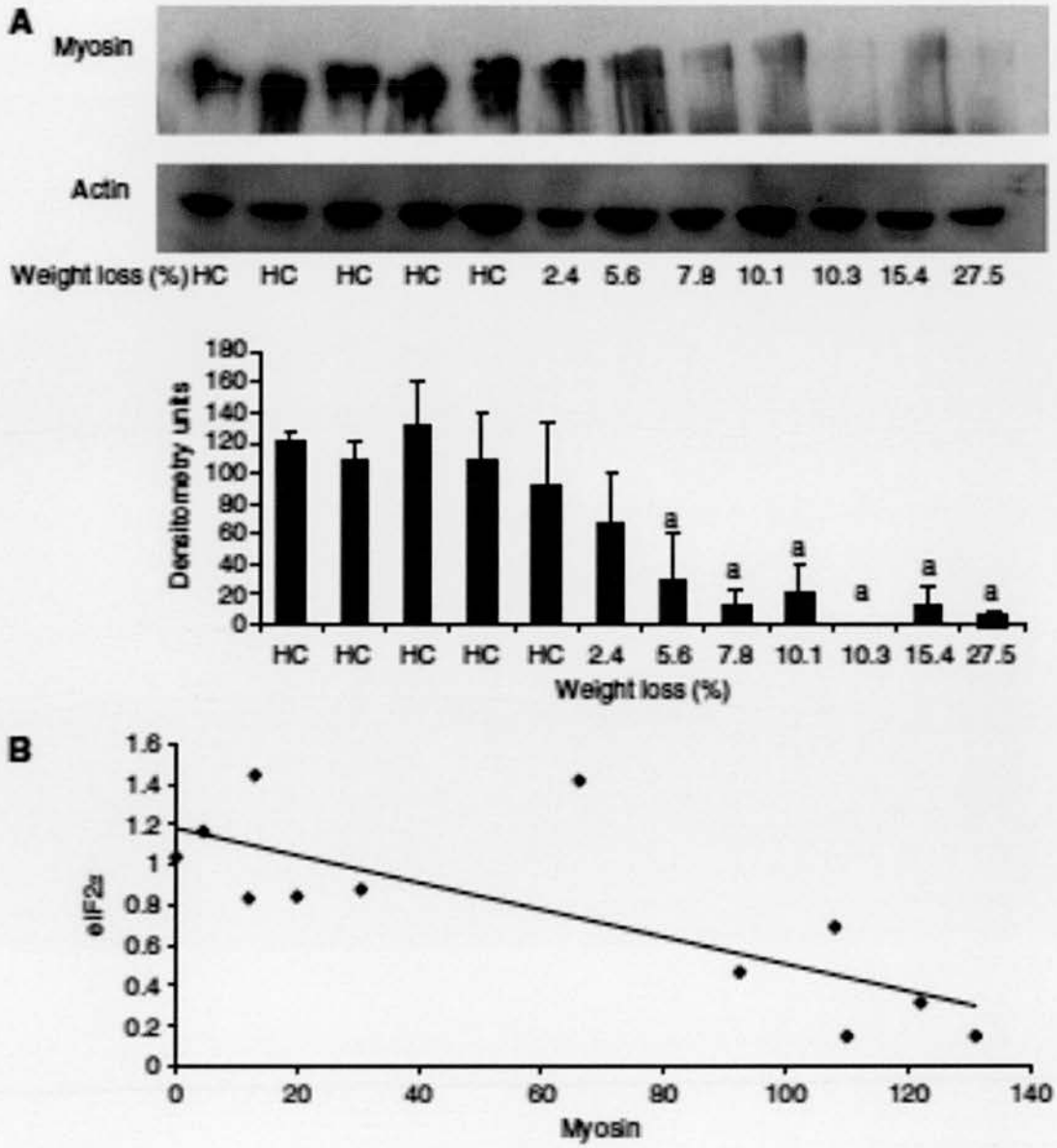

Figure 8.5 **(A) Western blot of myosin expression in rectus abdominis muscle of the patients shown in Figure 8.3 as a function of weight loss.** Myosin (MyHC II) expression was reduced in weight-losing cancer patients. Actin was used as a loading control. Each lane represents muscle from an individual patient. Densitometric analysis represents the average of three separate blots. Differences from healthy controls are shown as a, $p<0.05$.

(B) Relationship between the level of myosin and phospho eIF2α in skeletal muscle samples from patients shown in Figure 8.5A. There was a negative linear correlation between the expression levels of myosin (MyHC II) and phospho eIF2α ($r=-0.77$, $p=0.004$).

There was an inverse linear relationship between myosin expression and the extent of phosphorylation of eIF2α ($r=-0.77$, $p=0.004$) (Figure 8.5B), suggesting that phosphorylation of eIF2α was associated with reductions in myosin expression.

8.6 Discussion

This is the first study to show an increased expression of phosphorylated PKR and eIF2α in the skeletal muscle of weight-losing cancer patients, compared with healthy weight-stable controls. As found in the gastrocnemius muscle of weight-losing mice bearing the MAC16 tumour [188], expression of both phospho PKR and eIF2α was increased in cancer patients with weight loss. This suggests that the same signalling mechanism is operative in the skeletal muscle of both cachectic cancer patients and mice with experimental cancer cachexia. Similar findings have been observed in murine myotubes in the presence of murine PIF or angiotensin II and are thought to be responsible for the depression in protein synthesis and increase in degradation [188].

PKR is normally activated in response to viral attack, and the depression of protein synthesis resulting from phosphorylation of eIF2α constitutes one of the major ways in which viral replication is impaired [642]. However, PKR can also exert effects in uninfected cells and can be a potent growth inhibitory protein when activated [643]. PKR is also linked to the induction of pro-apoptotic genes by dsRNA, and may trigger cell death in response to viral infection and possible tumourigenesis [644]. Activation of PKR by murine PIF may also be involved in its ability to induce apoptosis in murine myotubes [189]. Certainly, it has been suggested that activation of PKR by PIF and angiotensin II is mediated through Ca^{2+} induced activation of caspases-3 and -8 [190]. Furthermore, PKR has been linked to ROS production via p38MAPK in response to bacterial lipopolysaccharide [645]. Increased apoptosis has been observed in the skeletal

muscle of rats bearing the cachexia-inducing Yoshida AH-130 ascites hepatoma [646] and in the early stage of weight loss in rabbits bearing the VX2 carcinoma [647]. Thus, activation of PKR might be expected to contribute to wasting of the skeletal muscle of cachectic cancer patients by leading to increased rates of apoptosis. Phosphorylation of PKR would also be expected to lead to an increased breakdown of myofibrillar proteins in skeletal muscle by induction of the UPP [174, 640] through activation of NF-κB [641], analogous to the effect of murine PIF and angiotensin II [188].

The increased phosphorylation of eIF2α is likely to contribute to the depression of protein synthesis in the skeletal muscle of cancer patients, through the inhibition of eIF2B and subsequent translational repression [639], as was observed previously in murine myotubes treated with murine PIF and angiotensin II [188]. Phosphorylation of eIF2α has also been shown to be responsible for the inhibition of protein synthesis in rat liver by vasopressin [648] and rat skeletal muscle by IL-1 [649]. The observed inverse linear correlation between intramuscular myosin protein levels and phospho eIF2α would support the concept of a direct role for eIF2α phosphorylation in the suppression of myofibrillar protein synthesis in cancer. Furthermore, the linear relationship between activation (autophosphorylation) of PKR and phosphorylation of eIF2α suggests that PKR is responsible for this effect rather than general control non-repressed 2 (GCN2 - the protein kinase usually considered to be in control of eIF2α phosphorylation), which one might expect to be activated [650] by the reduction in plasma levels of amino acids in cachectic subjects [651].

In the absence of conclusive evidence for the existence of human PIF (see Chapter 3, p.199, and Chapter 4, p.218), the identity of the key initiator of PKR phosphorylation in human muscle wasting is unclear. Angiotensin II remains a possible candidate, but at present, there is also a paucity of human data linking this potential mediator to cancer cachexia. Genetic studies have shown alterations in body composition in advanced cancer patients in relation to ACE gene polymorphism status [177], but further human data are required to ascertain the clinical implications of ACE in cancer cachexia.

In the current study, although expression of both phospho PKR and phospho eIF2α was increased in cancer patients with weight loss, there was no consistent trend towards increased expression with increasing weight loss. In contrast, in studies using murine MAC16 cachexia model, these changes appeared to be progressive [188]. However, most of the recruited patients were at a relatively early stage of disease where resectional surgery was still considered possible. Moreover, although some patients had lost a considerable amount of weight, no patients were below the normal BMI range (20-25 kg/m^2). (This reflects, in part, the general trend towards obesity in the current general population and the fact that obesity is a risk factor for oesophago-gastric adenocarcinoma). Thus, it remains to be determined whether in patients with more advanced cachexia a similar pattern would be observed as seen in mice bearing the MAC-16 tumour.

In the MAC-16 model, treatment with a PKR inhibitor, at a concentration that reduced levels of phospho PKR down to that found in non tumour-bearing animals,

effectively attenuated the depression of body weight, through an increase in LBM [652]. This was achieved through attenuation of both the depression in protein synthesis and the increase in protein degradation observed in murine myotubes exposed to either PIF or angiotensin II [188]. Thus, pathological human muscle wasting may also be responsive to inhibitors of PKR. Such wasting might include that experienced by cachectic cancer patients, but also patients with other pathological disorders. For example, angiotensin II has been linked with muscle wasting in CHF [653]. TNF-α, which may be linked to muscle wasting in sepsis, AIDS and parasitic infections, as well as cancer, has also been shown to activate PKR [654]. Furthermore, burn injury is associated with increased phosphorylation of eIF2α as a result of a 274% increase in phosphorylation of PKR [655].

This chapter has demonstrated that phosphorylation of PKR and eIF2α are key events seen in human muscle wasting, and that these events are proposed as mechanisms leading to a repression of protein synthesis and an increase in protein degradation. Thus, intramuscular levels of phospho PKR and phospho eIF2α could, in the future, be investigated as potential biomarkers of cachexia in human trials. This chapter concludes Part C concerning molecular mechanisms of muscle wasting, but the supposition regarding biomarkers leads appropriately into the focus of the next chapter (Part D), which explores specifically the use of urinary proteins as biomarkers of skeletal muscle wasting in human cancer cachexia.

Part D – Biomarkers of Muscle Wasting in Cancer Cachexia

Chapter 9 - Mass spectrometric detection of candidate protein biomarkers of cancer cachexia in human urine

9.1 Introduction

In Chapters 7 and 8, molecular mechanisms leading to protein degradation and skeletal muscle wasting in human cancer cachexia have been described. During this catabolic response in skeletal muscle, release of myofilaments from the sarcomere by calcium/calpain-dependent pathways may be an early, and perhaps rate-limiting, step [656]. Activation of the UPP is then believed to degrade the released myofibrillar proteins [225]. In murine tumour models, cancer cachexia involves the selective reduction of MyHC protein expression within skeletal muscle [294]. This reduction in MyHC levels is associated with increased activity of the UPP, suggesting that cachexia is the result of targeted depletion of specific myofibrillar proteins. Studies in cytokine-dependent animal tumour models have also identified specific changes in skeletal muscle membrane structure and function during cachexia [321]. In particular, the DGC appears deregulated and the sarcolemma becomes permeable to vital dyes. In Chapter 7, similar deregulation of the DGC was identified in cachectic human muscle. The severity of membrane damage would appear, at first glance, to be insufficient to cause a free permeation of intracellular proteins, particularly as circulating levels of CK, a marker of muscle damage, are not elevated [321, 657]. However, it was hypothesised that highly permeable cachectic muscle fibres might excrete some products of muscle protein degradation, which then enter the circulation, are filtered by the kidneys, and are

expelled in patient urine. The multifactorial catabolic and inflammatory mechanisms at work in cancer cachexia are already known to result in an increase in circulating proteins, leading to significant proteinuria and urinary nitrogen loss [658]. Such proteinuria has been shown to be associated with worsened patient prognosis [659]. However, the exact identity of protein species within urine has not been elucidated.

The systematic approach to the management of cachexia requires early identification of patients at risk and institution of prophylactic measures to attenuate its progression. However, there are currently no agreed early biomarkers (clinical or biochemical). It was hypothesised that the identification of proteins within the systemic compartment of cachectic patients may identify proteins that have been degraded selectively and expelled by "leaky" cachectic muscle, and that may thus act as biomarkers of muscle wasting. The identification of proteins within patient plasma (as a representative matrix of the systemic compartment) is highly complex due to the multiplicity of species involved in non-cachectic processes found therein [660]. However, MS has been successful at species identification within urine [661]. Thus, in a preliminary attempt to identify urinary proteins that might be used in future biomarker studies to target patients for therapeutic intervention, the aim of the present study was to use MS to identify and compare the protein contents of urine from cachectic cancer patients, weight-stable cancer patients and healthy controls.

9.2 Hypothesis

Using MALDI-TOF MS and/or LC-MS/MS, degraded skeletal muscle protein products can be detected in the urine of weight-losing patients with OGC, whereas such products are absent from the urine of weight-stable patients and healthy controls.

9.3 Patients and healthy controls

Patients with OGC and varying degrees of weight loss (n=16) were recruited (see Methods Chapter 2.1, p.167). Patients were defined as either cachectic (n=8) or weight-stable (n=8), depending on the presence or absence of cachexia (defined as weight loss $\geq$10%). Laboratory staff were recruited as healthy controls (n=8) for comparative analysis. Nutritional assessment was performed (see Methods Chapter 2.2, p.168). Urine samples were obtained and the urinary proteins were precipitated (see Methods Chapter 2.5, p.175). Proteins were then separated using 1D SDS-PAGE (see Methods Chapter 2.9.1, p.187). Following electrophoresis, gel blocks were excised and trypsinised (see Methods Chapter 2.9.2, p.188). Tryptic digests were then analysed by MALDI-TOF MS and LC-MS/MS (see Methods Chapter 2.9.3 and 2.9.4, p.188-189). Blood samples were also obtained and analysed for plasma CRP concentration (see Methods Chapter 2.3.3, p.170), creatinine concentration and CK concentration (see Methods Chapter 2.3.7, p.173). Exclusion criteria included known renal failure (defined as creatinine >120μmol/L in males or >110μmol/L in females or estimated glomerular filtration rate <60ml/min/1.73m^2).

9.4 Statistical analysis

Concentrations of the precipitated protein supernatants were used to derive protein concentrations of the initial urine samples in mg/L. Differences between distribution functions of data for the 3 patient groups was determined by KWT. Subsequent analysis to determine differences between any 2 groups was determined by MWT and FET. Asymptotic p-values are quoted for KW test whereas two-sided exact p-values are quoted for MW test.

9.5 Results

9.5.1 Patient demographics

Tumour site, histology, and stage of disease did not differ between the weight-stable and cachectic cancer groups (Table 9.1). The control group was younger than both the weight-stable cancer group (p=0.001) and cachectic cancer group (p=0.002) (Table 9.2). However, there was no difference in age or sex ratio between the weight stable and cachectic cancer groups. The cachectic group had lost a median of 17.9% of pre-illness weight (range 12.7-25.6) compared with both the weight-stable cancer and control groups, which had not lost weight (p<0.001). Other nutritional parameters (BMI, MAC, MAMC) were also reduced in the cachectic group compared with the weight-stable cancer group, consistent with a specific loss of skeletal muscle mass in the cachectic patients. The weight-stable cancer group exhibited an elevated BMI compared with controls, reflecting the role of obesity in the pathogenesis of oesophago-gastric adenocarcinoma.

9.5.2 Plasma levels of CRP, creatinine and CK

Plasma creatinine was elevated in one patient in the weight-stable cancer group suggesting mild renal impairment, but there was no overall difference between the weight-stable and cachectic cancer patients (p=0.234). Six (37.5%) cancer patients exhibited evidence of an acute phase response (plasma CRP>10mg/L), but there was no difference in CRP between the weight-stable and cachectic groups (p=0.613). Plasma CK was not elevated in any of the cancer patients and did not differ between the weight-stable and cachectic groups (p=0.083).

	Weight-stable Cancer (n=8)	Cancer Cachexia (n=8)	Overall (n=16)
Tumour Site			
Oesophageal	3	5	8
Gastric	5	3	8
Histology			
Adenocarcinoma	7	5	12
Squamous	1	3	4
Disease Stage			
I	4	3	7
II	1	0	1
III	1	4	5
IV	2	1	3

Table 9.1 **Tumour details of the oesophago-gastric cancer patients (n=16) in the urinary biomarker study.**
There were no significant differences in tumour site, histology and disease stage between the weight-stable and cachectic cancer groups.

Patient Group	n	Sex M:F	Age [1] yrs	Weight Loss [1] %	Weight kg	BMI [1] kg/m^2	MAC [2] cm	TSF mm	MAMC [3] cm	KPS	Creat µmol/L	CK U/L	CRP mg/L	UPC mg/L
Healthy Controls	8	6:2	31.5 (21-59)	0 (0.0-0.0)	73.7 (62.8-82.9)	23.7 (23.0-28.6)	30.8 (29.9-34.5)	16.0 (8.4-26.6)	26.0 (21.5-31.6)	100 (100-100)	N/A	N/A	N/A	174.6 (74.7-343.5)
Weight Stable Cancer	8	7:1	72 [a] (56-74)	0 (-1.67-1.00)	73.0 (66.8-111.0)	32.0 [b] (25.3-33.5)	31.3 (29.0-35.2)	13 (6.2-25.8)	26.7 (24.4-32.1)	100 (90-100)	98 (76-193)	79 (22-128)	11 (<5-39)	105.3 (35.1-202.8)
Cancer Cachexia	8	5:3	70 [a] (48-82)	17.9 [a,d] (12.7-25.6)	52.2 [a,f] (39.6-90.0)	20.4 [c,d] (15.5-25.2)	24.3 [b,e] (18.0-31.0)	8.6 (3.0-17)	21.2 [c,e] (15.9-26.9)	100 (80-100)	90 (66-108)	43 (24-68)	5 (<5-47)	157.2 (65.4-288.9)

Table 9.2 **Demographics of the healthy controls, the weight-stable cancer patients and the cachectic cancer patients.**
The cachectic cancer group had lost a median of 17.9% of pre-illness weight (range 12.7-25.6) compared with both the weight-stable cancer and healthy control groups, which had not lost weight ($p<0.001$). Other nutritional parameters (BMI, MAC, MAMC) were also reduced in the cachectic cancer group compared with the weight-stable group, consistent with a specific loss of skeletal muscle mass in the cachectic cancer group. Data are presented as medians with ranges in parentheses.
BMI = body mass index; CK = creatine kinase; Creat = creatinine; KPS = Karnofsky performance score; MAC = mid-arm circumference; MAMC = mid-arm muscle circumference; NA = not assessed; TSF = triceps skinfold thickness; UPC = urinary protein concentration.
[1,2,3] = difference between the 3 groups on KW test are shown as $p\leq0.001$ [1]; $p<0.01$ [2]; $p<0.05$ [3].
[a,b,c] = difference from HC group on MW test $p\leq0.001$ [a]; $p\leq0.01$ [b]; $p<0.05$ [c].
[d,e,f] = difference from WS group on MW test $p\leq0.001$ [d]; $p<0.01$ [e]; $p<0.05$ [f]

9.5.3 Cachectic cancer urine contains more protein species than weight-stable cancer and control urine

There was no significant difference in the urinary protein concentration of the three groups (p=0.149) (Table 9.2). Thus, the finite amounts of protein loaded onto gels were comparable statistically. Using both MS approaches, the number of proteins identified in each cachectic urine sample (median 42, range 26-61) was greater than that identified in each weight-stable cancer sample (median 15; range 9-28) and control sample (median 12.5; range 5-18) ($p<0.001$) (Table 9.3). Furthermore, the total number of different protein species identified in all samples of the cachectic group combined (n=199) was higher than that identified in the weight-stable cancer (n=79) and control (n=49) groups (Table 9.3). There was no difference between the numbers of proteins identified in the weight-stable samples compared with controls (p=0.234). In all 3 groups, the number of proteins identified by MALDI-TOF MS was higher than that identified by LC-MS/MS.

9.5.4 Candidate biomarkers of cachexia

The distribution of gel bands did not differ observably between the 3 patient groups (Figure 9.1). Thirty-five protein species were identified that appeared in at least 3 urine samples within any patient group of 8 (i.e. at least 37.5%). Of these 35 species, 21 were not specific to the cachectic group. These non-cachexia-specific protein species included common urinary proteins such as albumin, cytokeratins 1 and 10, and uromodulin, plus many proteins not previously reported in the urine of cancer patients (Tables 9.4 and 9.5). Immunoglobulin (Ig) κ-light chain was identified in 6 (75%) cachectic samples and 4 (50%) weight-stable cancer samples

Patient Group	n	Urinary Proteins Identified			
		Number	MALDI-TOF[1]	LC-MS/MS	Combined[1]
Healthy Controls	8	Total	37	23	**49**
		Median	8.5	7	12.5
		Range	(5-16)	(3-11)	(5-18)
Weight Stable Cancer	8	Total	64	21	**79**
		Median	11.5[a]	6	15
		Range	(6-23)	(4-10)	(9-28)
Cancer Cachexia	8	Total	187	18	**199**
		Median	41.5[b,c,d]	6.5	42[c,d]
		Range	(26-59)	(4-9)	(26-61)

Table 9.3 Number of urinary proteins identified within each patient group by each mass spectrometric technique.

The number of proteins identified in each cachectic cancer urine sample was greater than that identified in each weight-stable cancer sample and healthy control sample ($p<0.001$). Furthermore, the total number of different protein species identified in all samples of the cachectic cancer group combined (n=199) was higher than that identified in the weight-stable cancer (n=79) and healthy control (n=49) groups. Total number of proteins identified within a patient group are shown in bold. Median number of proteins identified within a single urine sample are shown in normal type with range in parentheses.

[1] = difference between the 3 patient groups on KW test $p<0.001$.

[a, b] = difference from LC technique within same patient group on MW test $p<0.05$ [a]; $p<0.001$ [b].

[c] = difference from healthy control group on MW test $p<0.001$.

[d] = difference from weight-stable group on MW test $p<0.001$.

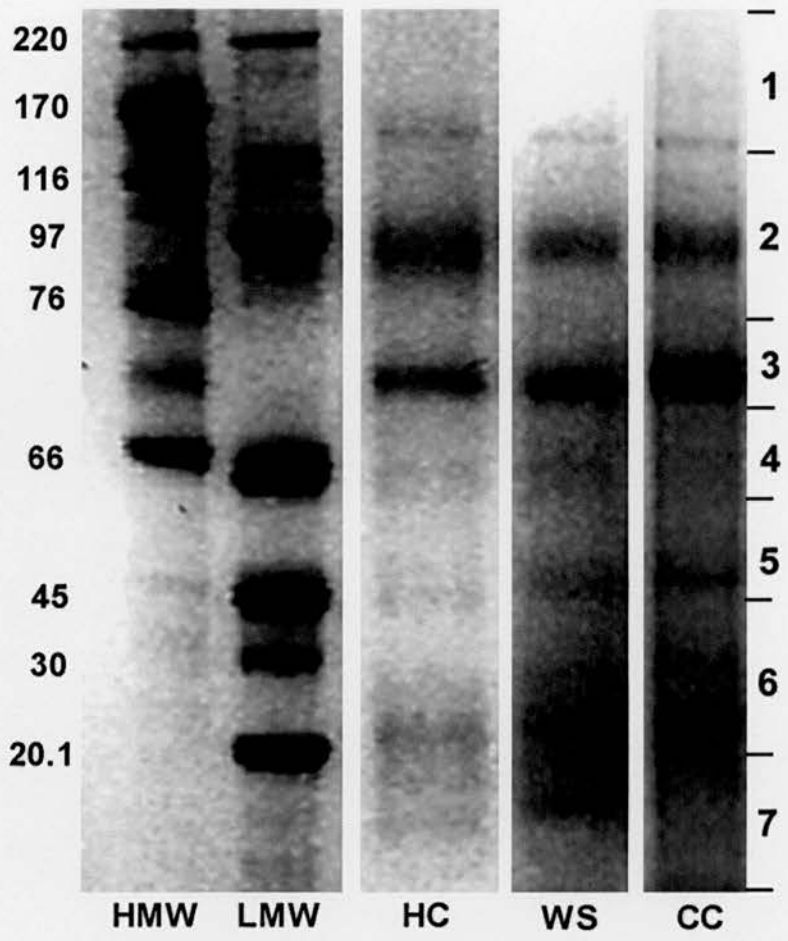

Figure 9.1 **Representative 4-12% Bis-Tris gradient gel lanes of urinary proteins from a healthy control (HC), weight-stable cancer patient (WS) and cachectic cancer patient (CC) with high (HMW) and low (LMW) molecular weight markers.**

The sites of gel block excision (blocks 1-7) for LC-MS/MS analysis and the locations of the abundant urinary proteins are demonstrated. Locations of the abundant urinary proteins: Block 1 – c-myc intron binding protein 1 (MIBP1), JmjC domain-containing histone demethylation protein 2A, maltase glucoamylase, microtubule associated protein 1B (MAP1B), myosins, spectrins, talin-2; Block 2 – chromosome 14 open reading frame 78; Block 3 – α-fetoprotein, uromodulin; Block 4 – albumin; Block 5 – kininogen, zinc-α-2 glycoprotein; Block 6 – α-1-microglobulin, microtubule actin crosslinking factor 1 (MACF1).

Protein	Accession Number	MW (Da)	MALDI Peptides	MALDI % Coverage	LC-MS/MS Peptides	Number of Individuals Excreting Protein (out of 8)		
						Healthy Controls	Weight-stable Cancer	Cancer Cachexia
Acetyl-CoA carboxylase 2	O00763	276555	21	12	N/A	2	3	0
α-1-Acid glycoprotein 1 precursor	P02763	23512	N/A	N/A	6	2	3	5
Albumin	P02768	69367	34	61	34	8	8	8
Centriolin	Q7Z7A1	268886	23	15	N/A	0	1	3
Collagen type VI, α3	P12111	343552	23	15	N/A	0	1	3
Cytokeratin 1	P04264	66018	18	38	10	5	5	4
Cytokeratin 10	P13645	59511	17	35	7	5	2	2
α-Fetoprotein	P02771	68678	33	56	3	6	5	7
Immunoglobulin κ light chain	Q6GMX8	25707	8	50	7	1	4	6 [a]
Leucine-rich repeat-containing protein KIAA1731	Q9C0D2	295146	20	14	N/A	3	1	0
Kininogen-1	P01042	71957	12	37	3	3	2	4

Table 9.4 **Protein species that were not cachexia-specific and were identified in at least three individual samples of any patient group of eight (I).**

Results reported as maximum number of peptides detected by MALDI-MS and LC-MS/MS within a single sample, and maximum peptide percentage coverage detected by MALDI-MS within a single sample.

[a] = difference from healthy control group on FE test $p<0.05$

Protein	Accession Number	MW (Da)	MALDI Peptides	MALDI % Coverage	LC-MS/MS Peptides	Number of Individuals Excreting Protein (out of 8)		
						Healthy Controls	Weight-stable Cancer	Cancer Cachexia
α-1-Microglobulin/bikunin	P02760	38999	17	58	12	7	7	7
Myosin 18B	Q8IUG5	285185	25	16	N/A	1	0	3
Neuroblastoma-amplified protein	Q8NFY8	268585	22	18	N/A	0	1	3
Probable G-protein coupled receptor 135	Q8IZ08	51736	N/A	N/A	1	5	2	0 [a]
Prostaglandin D2 synthase	Q5SQ09	22836	N/A	20	4	1	6 [a]	5
β-spectrin I (non-erythrocytic)	Q59ER3	274609	26	17	N/A	1	0	4
β-spectrin IV (non-erythrocytic)	Q71S07	289061	28	19	N/A	0	1	5 [a]
Talin-2	Q9Y4G6	271555	26	16	N/A	1	1	4
Uromodulin	P07911	69761	27	34	15	8	5	6
Zinc-α-2-glycoprotein	P25311	33872	21	61	11	1	5	5

Table 9.5 **Protein species that were not cachexia-specific and were identified in at least three individual samples of any patient group of eight (II).**

Results reported as maximum number of peptides detected by MALDI-MS and LC-MS/MS within a single sample, and maximum peptide percentage coverage detected by MALDI-MS within a single sample.

[a] = difference from healthy control group on FE test $p<0.05$

compared with 1 (12.5%) control sample (cachectic vs control p=0.041), whereas zinc alpha-2 glycoprotein (ZAG) (either precursor chains A-D or the whole molecule) was identified in 5 (62.5%) cachectic samples and 5 (62.5%) weight-stable cancer samples compared with 1 (12.5%) control sample. These two proteins may therefore represent markers of cancer or ill health.

All of the cachectic samples exhibited at least one species of myosin, including sarcomeric myosin heavy chain 2, and myosins 3A, 4, 5A, 5C, 7A, 8, 9A, 9B, 10, 13 and18B. In comparison, none of the weight-stable cancer samples expressed myosin species whereas only one (12.5%) control sample expressed myosin 18b. Furthermore, all of the cachectic samples expressed species of spectrin. Fourteen proteins were found exclusively in at least 3 out of 8 (37.5%) cachectic samples and may therefore represent specific markers of cachexia. All 14 of these proteins were detected by MALDI-MS, and not LC-MS/MS. The identities and functions of these proteins are shown in Tables 9.6 and 9.7. Retrospective analysis of 1D gels suggests that some markers (e.g. myosins and α-spectrin) were excreted in complete or near-complete form, whereas other biomarkers (e.g. MACF1) appear to have been digested into smaller peptides prior to excretion.

Candidate Biomarker Of Cancer Cachexia	Accession Number	MW (Da)	MALDI Peptides	MALDI % Coverage	Number of Individuals Excreting Protein (out of 8)		
					Healthy Controls	Weight-Stable Cancer	Cancer Cachexia
Bullous pemphigoid antigen 1 (isoforms 1-10)	Q03001	372210	22	18	0	0	3
Chromosome 14 open reading frame 78	Q8IVF2	616629	26	14	0	0	3
C-myc intron-binding protein 1 (MIBP1)	Q38G99	268803	20	16	0	0	3
Maltase-glucoamylase (intestinal)	O43451	209853	18	16	0	0	3
Microtubule-actin crosslinking factor (MACF1)	Q9UPN3	620418	49	16	0	0	3
Microtubule-associated protein 1B (MAP1B)	P46821	270620	21	15	0	0	5 [a]
Myosin 5C	Q9NQX4	202794	22	19	0	0	5 [a]
Myosin 7A	Q13402	254406	35	23	0	0	4
Myosin, heavy polypeptide 7, cardiac muscle, β variant	Q2M1Y6	223097	22	18	0	0	4
Myosin 9A	Q9UNJ2	292707	39	27	0	0	3
Myosin 10	P35580	228939	24	19	0	0	3
Nischarin	Q6PGP3	166653	18	20	0	0	5 [a]
α-Spectrin 1 (non-erythrocytic)	Q13813	284539	39	24	0	0	5 [a]
Zinc finger protein 106 homologue	Q9H2Y7	208883	27	20	0	0	6 [b]

Table 9.6 **Protein species that were specifically identified in at least three of the eight (37.5%) cachectic cancer urine samples and which therefore represent candidate biomarkers of cachexia.**

Results reported as maximum number of peptides and maximum percentage coverage found by MALDI-TOF within a single sample.

[a] = difference from both healthy control and weight-stable group on FE test $p<0.05$

[b] = difference from both HC and WS group on FE test $p<0.01$

Candidate Biomarker of Cancer Cachexia	Protein Function
Bullous pemphigoid antigen 1 (BPAG1) (isoforms 1-10)	Autoantigen of bullous pemphigoid (autoimmune subepithelial skin blistering disease). Member of spectraplakin family of cytoskeletal linker proteins. Highly expressed in skeletal muscle [657]. Anchors intermediate filaments to inner plaque of hemidesmosomes.
Chromosome 14 open reading frame 78	Unknown
C-myc intron-binding protein 1	Transcription factor. Belongs to the MHC binding protein family. May repress c-myc transcription from major promoter, P2 [658]. Knockout mouse demonstrates defects in T-cell maturation [659].
Maltase-glucoamylase (intestinal)	Determines small intestinal starch digestion into glucose. Alternate pathway for when luminal alpha-amylase activity is reduced due to immaturity or malnutrition [660]. May play role in digestion of malted dietary oligosaccharides used in food manufacturing.
Microtubule-actin crosslinking factor (MACF1)	Member of plakin family of cytoskeletal linker proteins [661]. Functions in microtubule dynamics to facilitate actin-microtubule interactions at cell periphery and to couple microtubule network to cellular junctions.
Microtubule-associated protein 1B (MAP1B)	Binds to tubulin to regulate microtubule function. Commonly found in dendrites and axons. Promotes axon formation through regulation of microtubule dynamics and cytoskeletal organisation. Muscle expression is increased in lean men by fat overfeeding [662].
Myosin 5C	Powers actin-based membrane trafficking, particularly of transferrin [663]. Expressed in many secretory and glandular tissues.
Myosin 7A	Expressed in testis, kidney, lung, inner ear, retina and the ciliated epithelium of nasal mucosa. Gene mutations are responsible for Usher syndrome type 1B [664], an autosomal recessive condition characterized by deafness and gradual vision loss.
Myosin, heavy polypeptide 7, cardiac muscle, β variant	Gene defects cause familial hypertrophic cardiomyopathy type 1 [665], myosin storage myopathy (involves type I fibre predominance and increased interstitial fat/connective tissue) [666], and Laing early-onset distal myopathy [667].
Myosin 9A	Expressed in all mammalian tissues. Involved in control of actin cytoskeleton by negatively regulating the small G-protein Rho [668]. Gene mutations implicated in Bardet-Biedl syndrome (autosomal recessive disorder characterized by cognitive impairment, obesity, retinitis pigmentosa, syndactyly/polydactyly, short stature, and hypogenitalism) [669].
Myosin 10	Involved in mitotic spindle function and filopodia formation [670]. Tail contains multiple pleckstrin homology domains [671].
Nischarin	Binds to cytoplasmic domain of integrin α5 subunit, inhibiting cell motility and altering actin filament organisation [672]. Inhibits Rac-induced migration and invasion of epithelial cells by affecting PAK signalling cascades [673].
α-Spectrin 1 (non-erythrocytic)	Cleavage of α-spectrin by calpain/caspase leads to membrane disruption and cellular death [674]. Overexpressed in some gastric tumours [675].
Zinc finger protein 106 homolog	Unknown

Table 9.7 **Functions of the candidate biomarkers of cachexia [662-680].**

9.6 Discussion

MS has been utilised successfully for the identification of urinary proteins [661], oligosaccharides [681] and nucleosides [682]. Furthermore, MALDI-MS has been employed in the detection of biomarkers in kidney disease [683], genitourinary tumours, including bladder [684] and prostate cancer [685], and tumours outwith the genitourinary tract, including pancreatic [686] and colon cancer [687]. In this preliminary study, MS was used to demonstrate that urine from cachectic OGC patients contained significantly more protein species than urine from weight-stable OGC patients and healthy controls, in the absence of an elevated CK level. Cancer patients were older than healthy controls, but ages of the cachectic and weight-stable cancer groups were comparable, and thus age alone is unlikely to explain the different numbers of protein species between groups.

MALDI-MS was capable of identifying more protein species than LC-MS/MS. Furthermore, all cachexia-specific protein species were detected exclusively by MALDI-MS. These results probably reflect the increased sensitivity of MALDI-MS, and the fact that, during LC-MS/MS analysis, highly abundant proteins will mask less abundant species [688]. Masking may have taken place during LC-MS/MS analysis within the present study as, for example, uromodulin was detected by LC-MS/MS in all members of the control group, but was found in only 2 of the weight-stable cancer group and 1 of the cachectic patients.

The protein species identified specifically in the cachectic urine samples could be considered to share a common structural function, and thus their urinary excretion

might point to a *prima facie* deterioration in muscle structure during cachexia. The myofibrillar proteins detected in cachectic urine were predominantly myosin species. Titin and cytoplasmic actin 1 were found in individual cachectic patients but none of the other core myofibrillar proteins, such as troponin T and tropomyosin, were detected. Myosins can be subdivided into 18 different classes based on homologous myosin head domain sequences [689]. Conventional myosins, such as those found in muscle tissue, are composed of two MyHC subunits, each associated with two light-chain subunits that bind at the neck region and play a regulatory role. Unconventional myosins, believed to function in intracellular motion and signal transduction [690], may contain one or two heavy chains, and associated light chains. The seven MyHC isoforms that predominate in mammalian skeletal muscles include two developmental isoforms (MyHC-embryonic and MyHC-perinatal); three adult skeletal muscle isoforms (MyHC-IIa, MyHC-IIb, MyHC-IIx/d); and MyHC-β/slow, which represents the main MyHC component in slow, oxidative, type I skeletal muscle fibres and cardiac muscle (24). This last isoform (specifically MyHC 7/cardiac muscle/β variant) was identified specifically in cachectic urine within the present study. At first glance, this result might appear contradictory to previous studies (including Chapters 7 and 8), which have predominantly found a reduction in type II fast fibres in cachectic murine and human muscle [321] (although expression levels of MyHC I were not tested specifically in Chapter 8). However, Acharyya *et al* also found slight reductions in type I MyHC within cachectic muscle [321], whereas other studies have demonstrated switching of fibre type from I to IIa in animal tumour models [691].

The remaining four myosins (5C, 7A, 9A and 10) detected exclusively in cachectic patients were all unconventional myosins. Myosin 18B was detected in 3 cachectic patients, but was not cachexia-specific as it was also found in one healthy control. Myosin 18B is detected predominantly in all types of striated muscle but at much lower levels compared to class II sarcomeric myosins. It moves into the myonuclei on differentiation to possibly regulate muscle-specific genes [692].

The cytoskeletal proteins detected in cachectic urine included α-spectrin 1 (non-erythrocytic) and nischarin. The β spectrins I and IV were also found in several cachectic samples but were not specific. The spectrins are a family of widely distributed filamentous cytoskeletal protein heterodimers that consist of a constant alpha-chain and variable, tissue-specific beta-chains, which associate with short actin filaments to form a hexagonal mesh. They play an important role in the maintenance of plasma membrane integrity [679]. In pathological processes such as diffuse axonal injury, cleavage of α-spectrin by calpain/casapse activity (an enzyme system hypothesised to be involved in the early stages of myofibrillar degradation) leads to membrane disruption and, ultimately, cellular death [679], suggesting that the cachectic process does indeed involve membrane disruption. α-Spectrin has been found to be overexpressed in intestinal-type gastric tumours [680], but in the present cohort was found primarily in samples from patients with oesophageal cancer. Nischarin binds to the cytoplasmic domain of the integrin α5 subunit, thus inhibiting cell motility, and altering actin filament organisation [677].

Another high-molecular weight cytoskeletal protein involved in integrin function and found in a high proportion, albeit non-specifically, within the cachectic samples was talin-2. Talin is concentrated at regions of cell–substratum contact and is capable of activating integrins enabling linkage to the actin cytoskeleton [693]. It is also a substrate for the calpain system [694].

The microtubule-associated proteins found specifically in the cachectic samples included microtubule associated protein 1B (MAP1B) and microtubule actin crosslinking factor 1 (MACF1). Microtubule-associated proteins (MAPs), including MAP1B, are thought to regulate the formation and stability of microtubules via their interaction with tubulin [695]. MACF1 is a member of the plakin family of cytoskeletal linker proteins and is a structural hybrid of dystrophin (at the C-terminal) and bullous pemphigoid antigen 1 (BPAG1) (at the N-terminal) [666]. BPAG-1 is another member of the plakin family which is highly expressed in skeletal muscle [662] and which was also identified specifically in the cachectic cancer group.

Two-thirds of the cancer samples expressed Ig κ-light chain and/or ZAG. Ig light chains have been detected in the urine of ill patients since 1848 (so-called Bence-Jones protein). Recently, Ig κ-light chains have been demonstrated in the urine of patients with CHF and various malignancies [208]. ZAG has been found previously in increased quantities in the urine of patients with bladder cancer [696]. Furthermore, ZAG has been postulated as the lipid mobilising factor in cachexia

[221]. However, a putative human homologue of PIF [17] was not detected in any of the urine samples.

One disadvantage of the present study is that it has utilised probability-based database searching. Thus, the identified urinary proteins represent statistical predictions based on smaller peptide fragments. Such a methodology also gives rise to the risk of redundancy within the identified biomarkers. Some MS studies have suggested that the urinary proteome may contain as many as 1500 species [697]. Only a third of proteins identified are described as classical circulatory plasma proteins, which are known to be relatively abundant in urine despite their retention to a large extent during the glomerular filtration process. However, other studies have demonstrated that sequential preparation of urine samples by gel filtration and protein precipitation results in low amounts of detectable protein on 2D gels [698]. These latter results are consistent with the present findings. Improvements in methodology, such as the use of storage agents [699]; alternative strategies of protein precipitation [700]; a return to 2D PAGE [701]; or the use of bioinformatics [701] may all increase the accuracy and yield of potential biomarkers.

In the present study, weight loss alone was used as the definition of cachexia. It has been shown previously that a 3-factor profile of cancer cachexia, incorporating weight loss, dietary intake and systemic inflammation, better defines groups with worsened prognostic outcome [8]. Future biomarker studies that incorporate more detailed definitions of cachexia will help to elucidate changes caused independently by systemic inflammation and hyponutrition.

In summary, it would appear that degraded skeletal muscle protein products can be detected in cachectic cancer patient urine using MS techniques. These molecules are candidates for potential targeting as novel biomarkers of skeletal muscle wasting and cachexia in OGC patients. Biomarker strategies targeting myofibrillar, cytoskeletal and microtubule-associated proteins within the context of future longitudinal studies of large patient numbers will be required to assess fully the changing prevalence of biomarkers within different cachectic populations.

In the next chapter (Part E), the study of biomarkers for use in clinical trials continues. However, rather than focus on molecular markers of skeletal muscle wasting, the use of objective assessment of PA as both a biomarker of skeletal muscle function and an outcome measure of cancer cachexia is explored.

Part E – Biomarkers of Muscle Function in Cancer Cachexia

Chapter 10 – Criterion-based validation of accelerometer-based activity-monitoring using a stable isotope dilution technique

10.1 Introduction

Patients with advanced cancer often report a significant decline in PF that has a major impact on QoL [702]. Such loss of QoL may be more pronounced in patients with cancer cachexia [703]. When developing palliative therapies focused either on the tumour or its systemic effects, one challenge is to use patient-centered outcomes that are 'fit for purpose' and relate clinically to PF and QoL during everyday situations [704].

In routine practice or clinical trials, PF is measured by healthcare provider instruments [489, 535], which are of diagnostic value, but lack responsiveness to change following disease progression and interventions. PA, as an indicator of PF, is assessed traditionally by self-report. However, such tools are subjective, correspond only loosely with objectively measured activity [705], and may fail to recognise activity characteristic of frail populations [706]. Recently, objective measurement of daily PA has been proposed as an important patient-centered outcome in advanced cancer [495]. Crucially, it has been shown that PA variables correlate with some QoL scores in advanced cancer patients [380], and that PA can be improved by nutriceutical intervention in cachectic pancreatic cancer patients [277]. However, gold-standard PA assessments (e.g. stable isotope studies) can be complex, patient-intense, and expensive, and often provide limited detail regarding

different PA behaviours. In contrast, modern accelerometer-based activity-monitoring systems potentially offer a patient-friendly methodology of long-term PA assessment, which can be used easily in both the clinical and free-living environments. Activity-monitoring systems can estimate EE based on the amplitude and frequency of acceleration signals or on recognised activities [707]. Accurate identification of postures and transfers in healthy young adults [708], older adults [709], and persons with minor functional limitations [710, 711] by activity-monitoring systems has been demonstrated previously. Activity monitoring systems have also been trialled for the objective assessment of PA in cancer patients undergoing palliative chemotherapy [380]. However, recognition of PA from acceleration signals may be potentially more challenging in frail patients who walk slowly and have a cautious movement pattern [712].

The aim of the present study was to assess whether a small, lightweight activity-monitoring system (activPAL™) could be used as an objective measure of daily PA and EE in both advanced cancer outpatients and healthy adults. More specifically, the criterion-based validity of activPAL™ was tested with regard to TEE and EEA against DLW and indirect calorimetry. Although activPAL™ offers a range of potential PA outcome measures, TEE and EEA were chosen as the outcomes of choice for validation as only these represent global and complete assessments of PA. Other outcomes (e.g. steps/day) may only offer information on a particular aspect of PA.

10.2 Hypothesis

The activPAL™ meter records accurate measures of EE in advanced cancer patients and healthy controls, which can be validated by the combination of DLW and indirect calorimetry.

10.3 Patients and healthy controls

Patients with advanced OGC (n=7), and healthy subjects (n=10 assessments in 9 subjects) with a wide spectrum of PA (ranging from sedentary office workers to competitive athletes) were recruited (see Methods Chapter 2.1, p.167), in order to validate the activPAL™ meter across the full range of PA. Exclusion criteria included physical handicap, severe co-morbidity/metastases that grossly impaired mobility, or inability to complete the study protocol or surgery/radiotherapy/chemotherapy during the previous 4 weeks. Recruits wore the activPAL™ meter (see Methods Chapter 2.10.1, p.190) during a 2-week DLW protocol (see Methods Chapter 2.10.3, p.194). Urine samples were collected for CF-IRMS analysis of stable isotope elimination rates (see Methods Chapter 2.10.3.3, p.195) and calculation of TEE (see Methods Chapter 2.10.3.4, p.195). Recruits were required to collect and complete at least 7 days of activPAL™ data [713]. Indirect calorimetry was performed to assess REE (see Methods Chapter 2.10.2, p.192).

10.4 Statistical analysis

'TEE_{MET}' was defined as the average MET.hrs/day over a recording period. To derive 'EEA_{MET}', the number of MET.hrs awarded for non-activity (24MET.hrs/day) was subtracted from TEE_{MET}. METs are measured in kcal/kg/hr and thus, to allow comparison between DLW and activPAL™, data were reduced to kcal/kg/hr by dividing DLW and indirect calorimetry data (expressed as kcal/day) by 24xbody weight, whereas activPAL™ data (expressed in MET.hrs/day) was simply divided by 24. Variables expressed in kcal/kg/hr are denoted with by ^{1}.

Demographics are presented as means, SD and ranges, and dichotomous variables are presented as absolute numbers and percentages. Differences between groups were determined using Student's Independent Sample t-test using exact, two-sided p-values. Bland-Altman plots with 95% limits of agreement (LOA) and percentage absolute errors were used to assess agreement between methods expressed in absolute units. Bi-variate relationships were assessed by Pearson's product moment correlation and linear regression. Linear regression was also used to determine the contribution of the two independent variables predicted REE and EEA by ActivPAL™ ($EEA_{ActivPAL}$), on the dependent variable TEE by DLW (TEE_{DLW}).

10.5 Results

10.5.1 Patients and healthy subjects

Two recruited subjects did not complete 7 days of activPAL™ data during the 2-week DLW protocol and were therefore excluded retrospectively. Fifteen assessments were included (in 6 cancer patients and 9 healthy subjects). Cancer patients did not differ from healthy subjects in LBM or fat mass (Table 10.1). As expected, when assessed by DLW, cancer patients exhibited lower mean TEE (2321kcal/day vs. 3202kcal/day; p=0.044) and EEA (742kcal/day vs. 1609kcal/day; p=0.036) compared with healthy subjects (Table 10.2).

10.5.2 Resting energy expenditure

When expressed in relation to body weight, average measured REE^{l} for the entire study cohort was 0.84kcal/kg/hr (SD=0.12, range=0.63-1.14). This value equates to the EE of 1MET and differs from the hypothesised value of 1kcal/kg/hr. Predicted REE was derived by multiplication of 0.84kcal/kg/hr by 24xbody weight (Table 10.2). A Bland-Altman plot of the agreement between predicted REE (mean=1587 kcal/day, SD=259, range=1160-2054) and measured REE (mean=1614 kcal/day, SD=168, range=1280-1885) demonstrated a mean difference of 27kcal/day (SD=218; 95%LOA=-400, 454) or absolute percentage error of 1.7% (Figure 10.1).

10.5.3 Energy expenditure of activity

Median length of activPAL™ monitoring was 14 days (range=7-14). On regression analysis, step count accounted for 80.0% of the variation in EEA^{l}_{DLW} (in kcal/kg/hr) (p<0.001) (Figure 10.2). However, the relationship between time spent upright and

	M:F	Age (yrs)	Height (m)	Weight (kg)	BMI (kg/m^2)	LBM (%)	Fat mass (%)	KPS
Whole Cohort (n=14)	12:2	43 (19, 25-76)	1.75 (0.06, 1.66-1.84)	80.0 (12.8, 57.5-101.8)	26.2 (4.2, 20.4-33.5)	70.1 (9.4, 52.1-84.3)	29.9 (9.4, 15.7-47.9)	95 (8, 80-100)
Cancer Patients (n=6)	6:0	65[a] (7, 59-76)	1.72 (0.07, 1.65-1.82)	86.0 (11.5, 69.4-101.8)	29.0[b] (3.6, 25.3-33.5)	65.5 (5.3, 57.4-73.0)	34.5 (5.3, 26.6-42.6)	87[a] (8, 80-100)
Healthy Subjects (n=8)	6:2	28 (2, 25-31)	1.77 (0.06, 1.68-1.84)	76.0 (12.6, 57.5-94.6)	24.3 (3.5, 20.4-30.7)	73.2 (10.5, 52.1-84.3)	26.8 (10.5, 15.7-47.9)	100 (0, 100-100)

Table 10.1 **Demographics of the DLW study subjects (n=14).**
Cancer patients did not differ from healthy subjects with regard to lean body mass or fat mass. However, cancer patients did demnostrate lower KPS than healthy subjects. Values are means with SDs and ranges in parentheses.
BMI = body mass index; KPS = Karnofsky performance score; LBM = lean body mass.
[a] = difference from healthy controls on independent t-test $p<0.001$
[b] = difference from healthy controls on independent t-test $p<0.05$

	Indirect Calorimetry		Doubly Labelled Water			activPAL™				
	Measured REE (kcal)	Predicted REE (kcal)	TEE_{DLW} (kcal)	EEA_{DLW} (kcal)	PAL_{DLW}	EEA_{METs} (MET.hrs)	$EEA_{activPAL}$ (kcal)	TEE_{METs} (MET.hrs)	$TEE_{activPAL}$ (kcal)	$PAL_{activPAL}$
Whole Cohort (n=15)	1592 (168, 1280-1885)	1614 (259, 1160-2054)	2849 (849, 2017-5309)	1262 (807, 374-3424)	1.80 (0.47, 1.21-2.82)	9.8 (1.8, 7.4-13.8)	1245 (825, 486-3334)	33.8 (1.8, 31.4-37.8)	2859 (787, 2129-5045)	1.82 (0.62, 1.26-3.43)
Cancer Patients (n=6)	1579 (104, 1465-1770)	1736 (233, 1400-2054)	2321[b] (347, 2017-2795)	742[b] (374, 374–1275)	1.48[b] (0.25, 1.21-1.84)	8.3[a] (0.8, 7.4–9.4)	701[b] (235, 486-1133)	32.3[a] (0.8, 31.4–33.4)	2436 (343, 2143-2985)	1.41[b] (0.14, 1.26-1.61)
Healthy Subjects (n=9)	1602 (211, 1280-1885)	1533 (255, 1160-1909)	3202 (915, 2478–5309)	1609 (845, 615–3424)	2.01 (0.46, 1.33–2.82)	10.7 (1.7, 8.9–13.8)	1607 (887, 873-3334)	34.7 (1.7, 32.9–37.8)	3140 (888, 2129-5045)	2.09 (0.67, 1.50-3.43)

Table 10.2 Physical activity level and energy expenditure of the DLW study assessments (n=15).

Cancer patients exhibited lower mean TEE, EEA and PAL compared with healthy subjects.

Values are means with SDs and ranges in parentheses.

EEA = energy expenditure of activity; METs = metabolic equivalents; PAL = physical activity level; REE = resting energy expenditure; TEE = total energy expenditure.

[a] = difference from healthy controls on independent t-test $p<0.001$

[b] = difference from healthy controls on independent t-test $p<0.01$

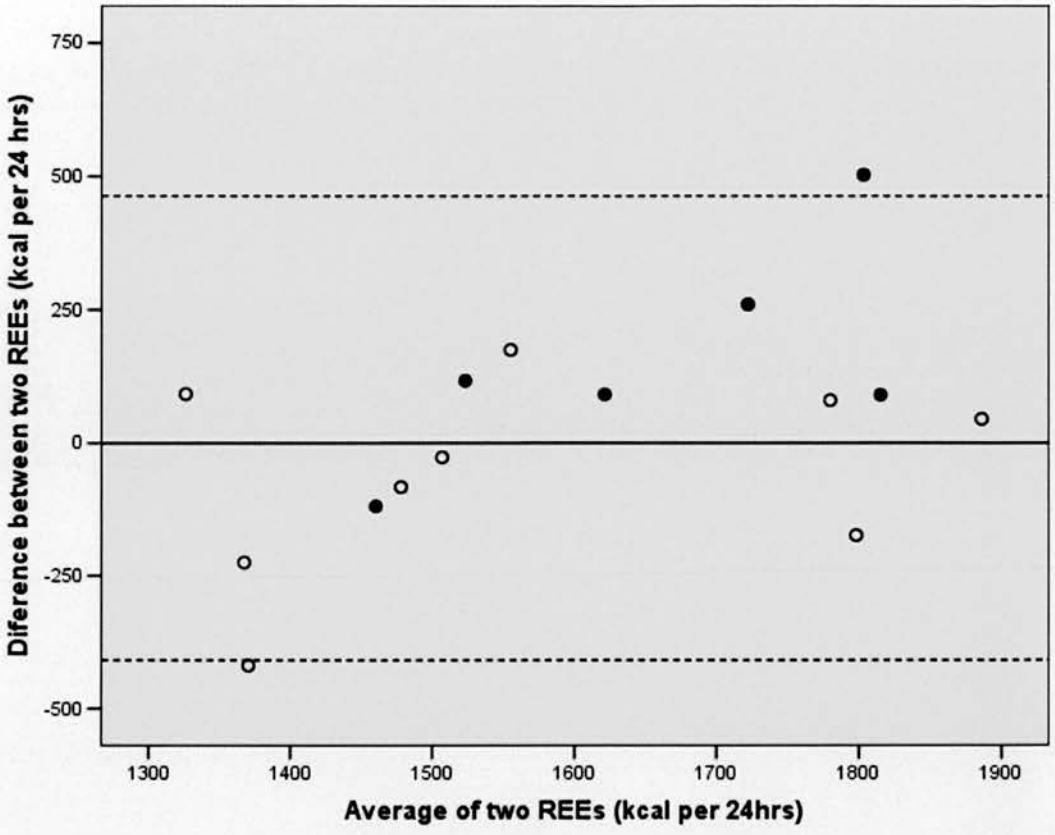

Figure 10.1 **Bland-Altman plot of agreement between $REE_{ActivPAL}$ and REE_{DLW}.**
Y axis defined by calculation: $REE_{ActivPAL}$ - REE_{DLW}. Dotted lines represent 95% limits of agreement (bias +/- 2SD). Black circles represent cancer patients whereas white circles represent healthy controls.
DLW = doubly labelled water; REE = resting energy expenditure.

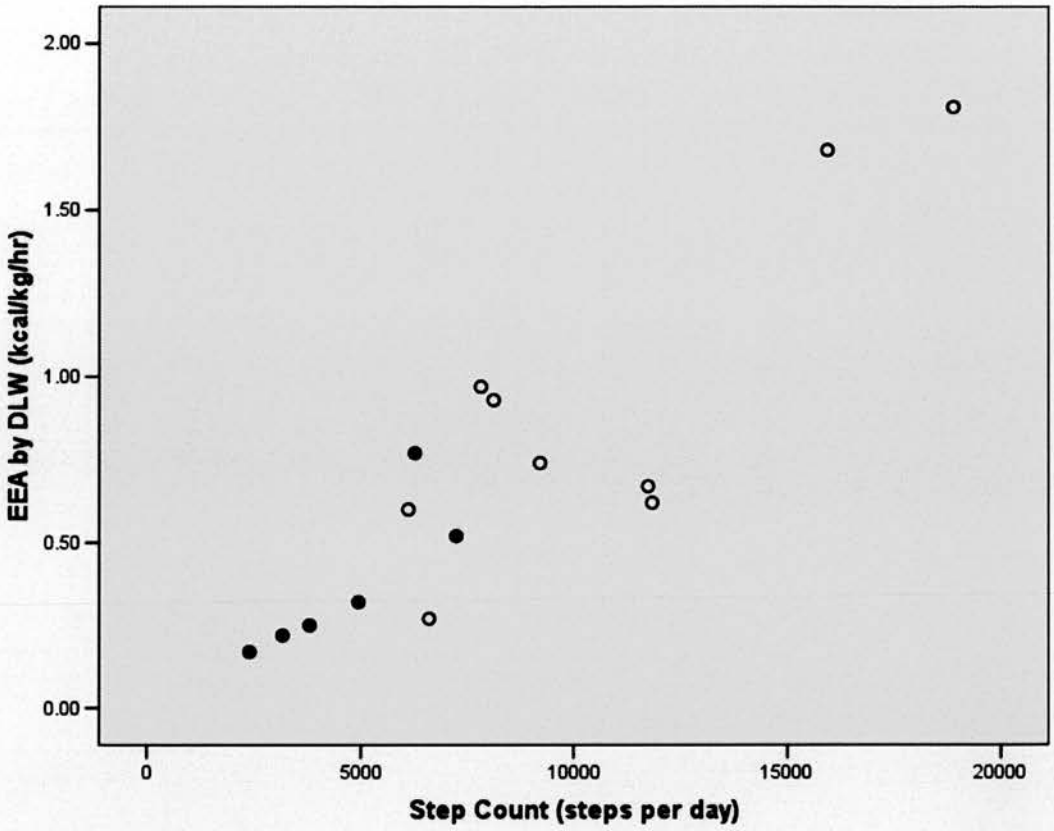

Figure 10.2 **Scatter plot of step count versus EEA by DLW (r^2=0.80).**
Black circles represent cancer patients whereas white circles represent healthy controls.
DLW = doubly labelled water; EEA = energy expenditure of activity.

EEA^{l}_{DLW} was less strong (r^2=0.52).

Values of EEA^{l}_{DLW} were higher than values of EEA^{l}_{MET} (Figure 10.3). These two variables expressed a non-linear relationship with equation: $EEA^{l}_{DLW}=15.08EEA^{l}_{MET}{}^{3.61}$ (r^2=0.80) (Figure 10.3). Thus, a validated estimate of EEA (in kcal/day) was derived using the equation:

$$EEA_{activPAL}=24 \times weight \times 15.08EEA^{l}_{MET}{}^{3.61}$$

This equation was transposed further to use the primary EE output of activPAL™, namely TEE_{MET}:

$$EEA_{activPAL}=362 \times weight \times ((TEE_{MET}-24)/24)^{3.61}$$

A Bland-Altman plot of the agreement between $EEA_{activPAL}$ (mean=1244kcal/day, SD=825, range=486-3334) and EEA_{DLW} (mean=1262kcal/day, SD=807, range=374-3424) demonstrated a mean difference of -18kcal/day (SD=347; 95%LOA=-699, 663) or absolute percentage error of 1.4% (Figure 10.4). Furthermore, as $TEE^{l}=REE^{l}+EEA^{l}$, a validated estimate of TEE (in kcal/day) was derived using the equation:

$$TEE_{activPAL}=24 \times weight \times [0.84+(15.08 \times ((TEE_{MET}-24)/24)^{3.61}]$$

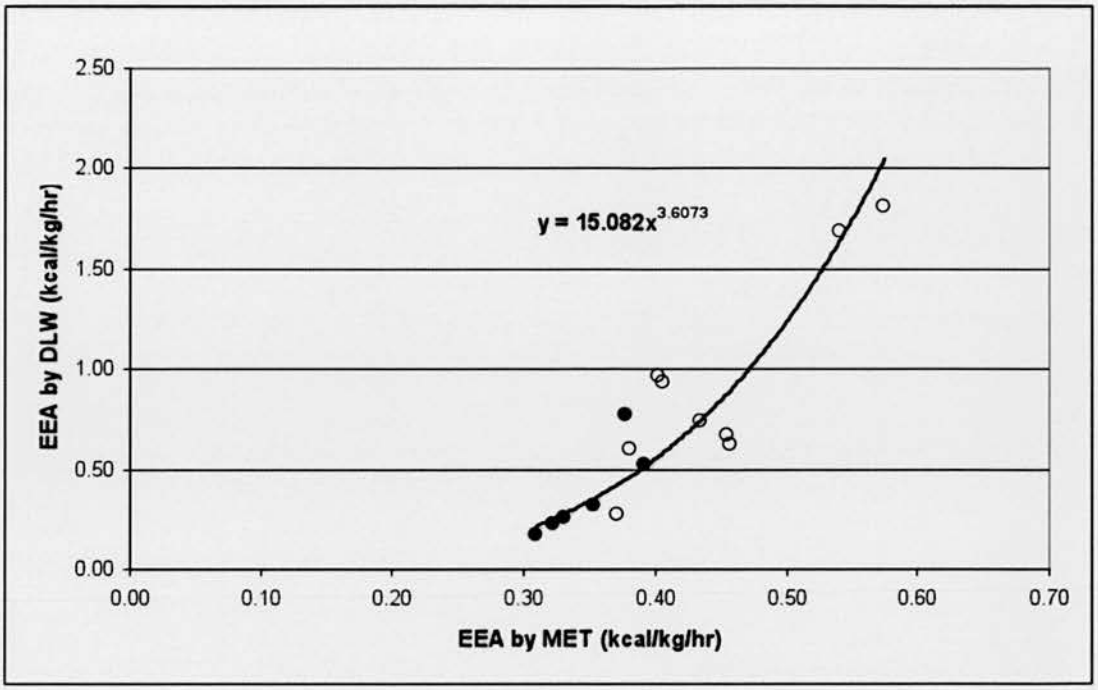

Figure 10.3 **Scatter plot of EEA^{1}_{MET} versus EEA^{1}_{DLW} (r^2=0.80).**
Black circles represent cancer patients whereas white circles represent healthy controls.
DLW = doubly labelled water; EEA = energy expenditure of activity; MET = metabolic equivalent.

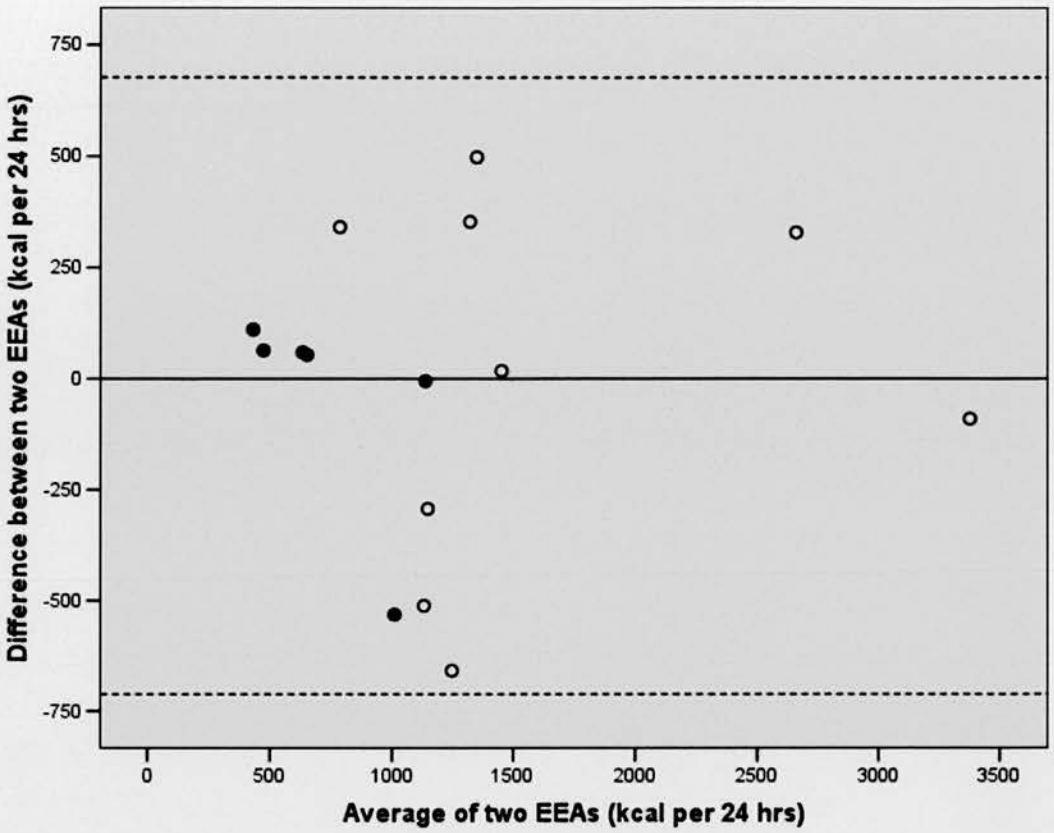

Figure 10.4 **Bland-Altman plot of agreement between $EEA_{ActivPAL}$ and EEA_{DLW}.**
Y axis defined by calculation: $EEA_{ActivPAL}$ - EEA_{DLW}. Dotted lines represent 95% limits of agreement (bias +/- 2SD). Black circles represent cancer patients whereas white circles represent healthy controls.
DLW = doubly labelled water; EEA = energy expenditure of activity.

A Bland-Altman plot of the agreement between $TEE_{activPAL}$ (mean=2859kcal/day, SD=787, range=2129-5045) and TEE_{DLW} (mean=2849kcal/day, SD=849, range=2017-5309) demonstrated a mean difference of 9kcal/day (SD=411; 95%LOA=-796, 814) or absolute percentage error of 0.4% (Figure 10.5). No obvious relationship was observed between TEE_{DLW} and the difference in TEE between the two methods as values were scattered evenly about the mean. In a regression model, $EEA_{ActivPAL}$ accounted for 85.1% of the variation in TEE_{DLW} ($p<0.001$), whereas predicted REE was not a significant determinant.

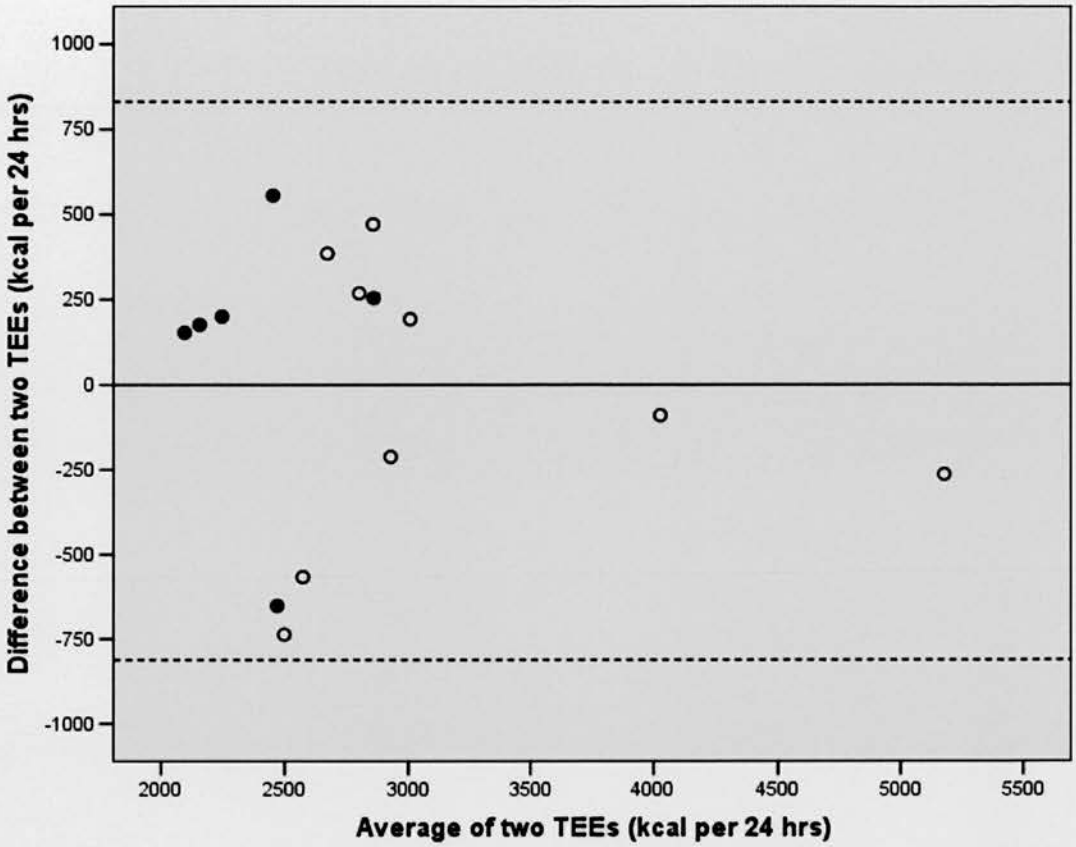

Figure 10.5 **Bland-Altman plot of agreement between $TEE_{ActivPAL}$ and TEE_{DLW}.**
Y axis defined by calculation: $TEE_{ActivPAL}$ - TEE_{DLW}. Dotted lines represent 95% limits of agreement (bias +/- 2SD). Black circles represent cancer patients whereas white circles represent healthy controls.
DLW = doubly labelled water; TEE = total energy expenditure.

10.6 Discussion

This study represents the first attempt to validate an activity-monitoring system in patients with advanced cancer. It demonstrates that mean percentage errors in the assessment of EE by the activPAL^{TM} meter in healthy controls and advanced cancer patients (with KPS 80-100) are low. However, to derive these small measurements of mean error, an element of mathematical correction was required. For measured EE variables, mean difference between the two methodologies (DLW and activPAL^{TM}) was small: mean bias in TEE between activPAL^{TM} and DLW was only 9kcal/day, whereas the mean bias in EEA between the two methodologies was an underestimation by activPAL™ of 18kcal/day. The results of the present study show either similar or superior mean agreement between activPAL™ and DLW compared with other studies of activity monitoring devices [714-717]. However, as retrospective mathematical correction was required, further studies are required to validate these measurements. Furthermore, despite small mean errors, within-subject variability was high. For example, when considering EEA, the mean measured EEA_{DLW} across the entire DLW study cohort was 1262kcal/day and yet the 95%LOA between the two methodologies was approximately +/-700kcal/day i.e. a potential variability of 55%. This high degree of variability would render the conclusions of any intervention study using activPAL™-derived EEA as an outcome measure very difficult to interpret. Sources of error that may have introduced disagreement between methods in the current study would have included under-report of steps during slow walking [712]. Recently, it has been confirmed that the activPAL™ meter is accurate in the registration of time spent in different body postures and the number of sit-to-stand transfers, but is inaccurate at step

count, in patients with advanced cancer (Guro Stene: personal communication from a study carried out by a Norwegian group as a companion study to the present one). These observations correspond with the body posture and transfer findings of other validation studies of activPAL™ in healthy older persons [709], suggesting that time in different body positions and numbers of transitions between positions are reliable outcomes from the activity meter, even in frail persons. However, step count in patients with advanced cancer demonstrated significant errors, particularly in patients with lower KPS and slower gait speed. This is because slow gait speed gives low acceleration amplitudes that may fail to be detected by the software system as steps. Inspection of raw activPAL™ acceleration data in advanced cancer patients has confirmed that steps registered by the monitor were left unrecognised following software calculations. Similar high step count errors have also been demonstrated in slow walkers (<0.8 m/sec) when using other activity-monitoring systems [712, 718-720], and cumulatively, these observations suggests that, at the present time, algorithms embedded in activity-monitoring softwares may not be sophisticated enough to detect accurately step count in frail and slow walkers.

These same data also highlight the potential difficulties of validating PA outcome measures in "mixed" populations. If step count might be altered by gait speed, it could be argued that, in the future, validation studies should only be performed in tightly grouped, homogenous cohorts of cancer patients. This argument might be levelled at the present study, as, in a deliberate attempt to recruit individuals with a wide range of EE, frail cancer patients and healthy controls were included within the same study cohort.

Although inaccuracy in step count is likely to have played a role in the widened LOA observed within the present study, it is unlikely to be the sole reason, as there were no obvious linear relationships between gold standard-derived measures of EE (i.e. DLW-derived measures) and disagreement between methodologies. Another potential source of variability may be differences in upper limb activity between subjects. ActivPAL^{TM} only records thigh activity and thus the relatively smaller contributions of arm activity to TEE and EEA are not assessed. The non-linear (power) relationship between EEA^{1}_{MET} and EEA^{1}_{DLW} could therefore be explained by a proportionally larger contribution of upper limb activity to EEA in fitter, more active individuals. However, it is again worth noting that Bland-Altman plots did not demonstrate any significant variations in bias by activPAL^{TM} at extremes of TEE and EEA.

Although cachectic cancer patients are known to exhibit higher REEs than healthy controls, inter-subject variation in nutritional status or basal metabolic rate is unlikely to explain observed differences in TEE or EEA between methodologies as, in the present study, cancer patients and healthy controls did not differ significantly with regards to weight, LBM, and measured REE. However, consistent with previous studies [277], cancer patients did exhibit lower mean TEE and EEA.

The present study raises questions regarding the best way in which to utilise PA outcome measures within the context of future intervention studies. Firstly, which PA outcome measure would be superior for use? This study did not aim to compare the validity of different outcomes derived from the meter. Thus, the type of PA

measure that may be the most sensitive to change following anti-cancer/anti-cachexia intervention is yet to be identified. Simple measures, such as step count, may still represent superior outcomes for clinical trials, even in the face of systematic under-reporting, compared with more complex, "combination" outcomes, such as time spent upright or EEA. Future clinical trials may need to assess a range of different outcomes from activity meters initially, in order to stratify their relevant importance. Secondly, in which patients should the PA outcome measure of choice be investigated? Recently, much interest has focused on the concept of 'pre-cachexia' [25], and the fact that any systematic approach to the treatment of cachexia requires early identification of patients at risk and institution of prophylactic measures to attenuate the progression of disease, prior to the development of significant weight loss. In the 'pre-cachectic' phase, patients are more likely to be physically active than later in the disease trajectory, and thus the accuracy of PA outcomes measured by activity-monitoring systems may be at their highest.

In conclusion, activity meters are easy to use in the clinical setting, are time efficient and cause minimal burden to patients. Furthermore, activity-monitoring systems provide data on promising patient-focused outcomes for clinical trials and the clinical management of patients with advanced cancer. ActivPAL™ can be used to derive estimates of EE but there is considerable variability in the results, which would be consistent, in part, with the described inaccuracy in step count in frail patients. Further prospective EE validation studies are required. The role of activity-monitoring systems should be supported in advanced cancer/cachexia

patients, but particularly in patients with early cachexia receiving prophylactic intervention to prevent further deterioration, as these more active patients might have the highest accuracy of PA detection.

The present chapter has focused on the validation of an activity-monitoring system with regards to the assessment of EE, arguably the most robust measure of global PA. However, in the next chapter, all of the PA outcomes of the activPAL™ meter are instead assessed in a group of advanced cancer patients undergoing palliative chemotherapy, in an effort to detect how anti-cancer treatment and disease longevity might impact on patient PA.

Chapter 11 - Physical activity as an objective measure of performance status and quality of life in upper gastrointestinal cancer patients undergoing palliative chemotherapy

11.1 Introduction

Depression, fatigue, nausea, anorexia, pain, disturbances in the sleep-wake cycle and other symptoms may all affect cancer patients undergoing palliative chemotherapy [721]. Furthermore, certain chemotherapeutic agents are known to worsen skeletal muscle depletion and thus exacerbate cancer cachexia. The result of all these symptoms in advanced cancer patients is a number of adverse features, including lower PS [8], impaired response to anti-cancer therapy [4], and, ultimately, shortened survival [4].

In any future prophylactic management strategy for cancer cachexia, it is likely that most patients will have been newly diagnosed with advanced disease. At this stage, many patients will not yet have experienced significant weight loss, and may be regarded as 'pre-cachectic' [25]. Such individuals are likely to be considered for palliative chemotherapy. However, there are only limited objective data describing in detail the functional consequences of advanced cancer, cachexia and palliative chemotherapy. The data that do exist suggest that patients reduce their overall PA in order to maintain energy equilibrium [277, 380]. However, for cancer patients living at home, 'non-exercise PA' (e.g. walking) is a significant component of daily

PA, which plays an important role in social interaction and independent living. Any reduction of non-exercise PA will thus impair independence and QoL.

In previous studies using the activPAL™ meter for one-off PA assessments of advanced upper GI cancer patients undergoing palliative chemotherapy (n=20), total energy expenditure was 8% lower, time spent upright was approximately two hours/day less and step count was 43% lower compared with healthy, age-matched controls [380]. TEE (in METs) and step count correlated significantly with the FACIT-F TOI ($r=0.59$, $p\leq0.009$) but not with the FAACT-TOI, EORTC QLQ-C30 Physical Functioning (PF), Fatigue or Global Health Status/QoL scores [380].

The aims of the present study were twofold. Firstly, to perform a longitudinal analysis of objective PA in advanced upper GI cancer patients undergoing palliative chemotherapy in order to investigate changes induced by treatment. Secondly, to further investigate the relationship between PA, PS, mood and QoL in a larger cohort of patient assessments.

11.2 Hypothesis

Objective measures of PA in advanced upper GI cancer patients are affected deleteriously by palliative chemotherapy, and these changes are detectable using the activPAL™ meter. PA measures correlate with PS, mood and QoL scores.

11.3 Patients and healthy controls

Patients with advanced (stage IV) OGC (n=16) commencing on palliative chemotherapy were recruited (see Methods Chapter 2.1, p.167). Exclusion criteria included physical handicap or severe co-morbidity/metastases that grossly impaired mobility. At commencement of treatment, the intention was for each patient to receive 6 cycles of chemotherapy. Patients wore the activPAL™ meter (see Methods Chapter 2.10.1, p.190) for 7 days at sequential time-points during chemotherapy until either treatment was completed or the decision was taken by oncology staff to discontinue treatment due to patient ill-health or deteriorating PS. All time-point assessments were performed during periods whilst the patient was living at home (i.e. not a hospital inpatient). Time-points were I) pre-chemotherapy/after 1st cycle (i.e. at the beginning of intended chemotherapy); II) after 2nd, 3rd or 4th cycles (i.e. during the middle of intended chemotherapy); and III) after 5th or 6th cycle (i.e. at the end of intended chemotherapy). If the patient completed 6 cycles of treatment and was able and willing to comply, a further time-point was performed 2 months following chemotherapy completion (time-point IV). At each time-point, nutritional assessment was performed (see Methods Chapter 2.2, p.168), and EORTC QLQ-C30, FAACT, FACIT-F and HADS questionnaires were completed (see Methods Chapter 2.11, p.196).

11.4 Statistical analysis

Due to the variability in validity of TEE and EEA between individual patients in Chapter 10, EE has only been assessed using activPAL™-derived METs in the present chapter (rather than attempt to convert METs to kcals). EEA has been

chosen as the main EE output of activPAL™, as in this context, TEE simply equals EEA + 30METs/day. EORTC QLQ-C30 data were reported and analysed using linearised scores. Data are presented graphically using box plots. Mild outliers (>1.5 times the IQR above the 3rd quartile/below the 1st quartile) are represented as circles and extreme outliers (>3 times the IQR above the 3rd quartile/below the 1st quartile) are presented as asterisks. Differences between multiple related groups were determined by the Friedman test (FT), whereas differences between any 2 related groups were performed using the Wilcoxon signed-rank test (WT). Differences between multiple independent groups were determined by the KWT, whereas differences between any 2 independent groups were performed using the MWT. All quoted p-values are two-sided asymptotic values except for MWT where two-side exact p-values are reported. Correlation analysis was performed using non-parametric Spearman's Rank Correlation Coefficient. When performing correlation analysis, LBM was assessed as a percentage of total body weight (rather than in kgs) in order to normalise LBM to body habitus. For observational analyses of pooled data (e.g. correlation analyses), all data from all time-points (I-IV) were included, but for longitudinal, time-series analyses, only data from time-points I to III were included. Reverse time-series analysis was performed by considering each patient's time-points in reverse order from the moment of cessation/completion of treatment. Thus, the time-point closest to cessation/completion of treatment was labelled time-point 1, the second time-point before the cessation/completion of treatment was labelled time-point 2, and so on. In this way, the higher the numbered label (1, 2 or 3) of the time-point, the longer the time duration before cessation/completion of treatment.

11.5 Results

11.5.1 Patients

The demographics and chemotherapy data of the recruited patients (n=16) are shown in Table 11.1. Five patients completed the full course of chemotherapy whereas 11/16 patients discontinued treatment early (median 4.5 cycles, range 2-6). Four patients performed the time-point I assessment only; 7 patients performed 2 sequential assessments; and 5 patients completed palliative chemotherapy and thus performed at least 3 sequential assessments (3/5 of these latter patients completed a further assessment at time-point IV). The total number of assessments was 36. A consort diagram of the chemotherapy patients is shown in Figure 11.1. Median percentage weight loss at time-point I was 8.3% (range 0.0-28.2).

11.5.2 Effect of palliative chemotherapy on physical activity

11.5.2.1 Longitudinal time-series analysis of individual patients

Patients demonstrated various PA responses following initiation of palliative chemotherapy (Figures 11.2a-d). For example, when considering EEA, of those patients who underwent sequential assessments at time-points I and II (n=12), 7 patients demonstrated deterioration in EEA, 4 patients demonstrated an elevation in EEA and 1 patient remained static. Interestingly, of the 4 patients that demonstrated an elevation in EEA, treatment was discontinued in 3 of them, and thus they did not have subsequent assessments. There were no obvious differences in PA between different chemotherapy regimens.

	Patients (n=16)
Age (yrs)	62 (43-83)
Sex (M:F)	12:4
Tumour Site	
Oesophageal	7
Gastric	7
Peri-ampullary	1
Unknown	1
Chemotherapy Regimen	
ECF	7
Oxaliplatin/Capecitabine	2
CF	2
ECX	2
MCX	2
Cisplatin/Gemcitabine	1
Chemotherapy Completion (Yes:No)	5:11

Table 11.1 **Demographics and chemotherapy regimens of the recruited palliative chemotherapy patients (n=16).**
C = cisplatin; E = epirubicin; F = 5-FU; M = mitomycin C; X = capecitabine.

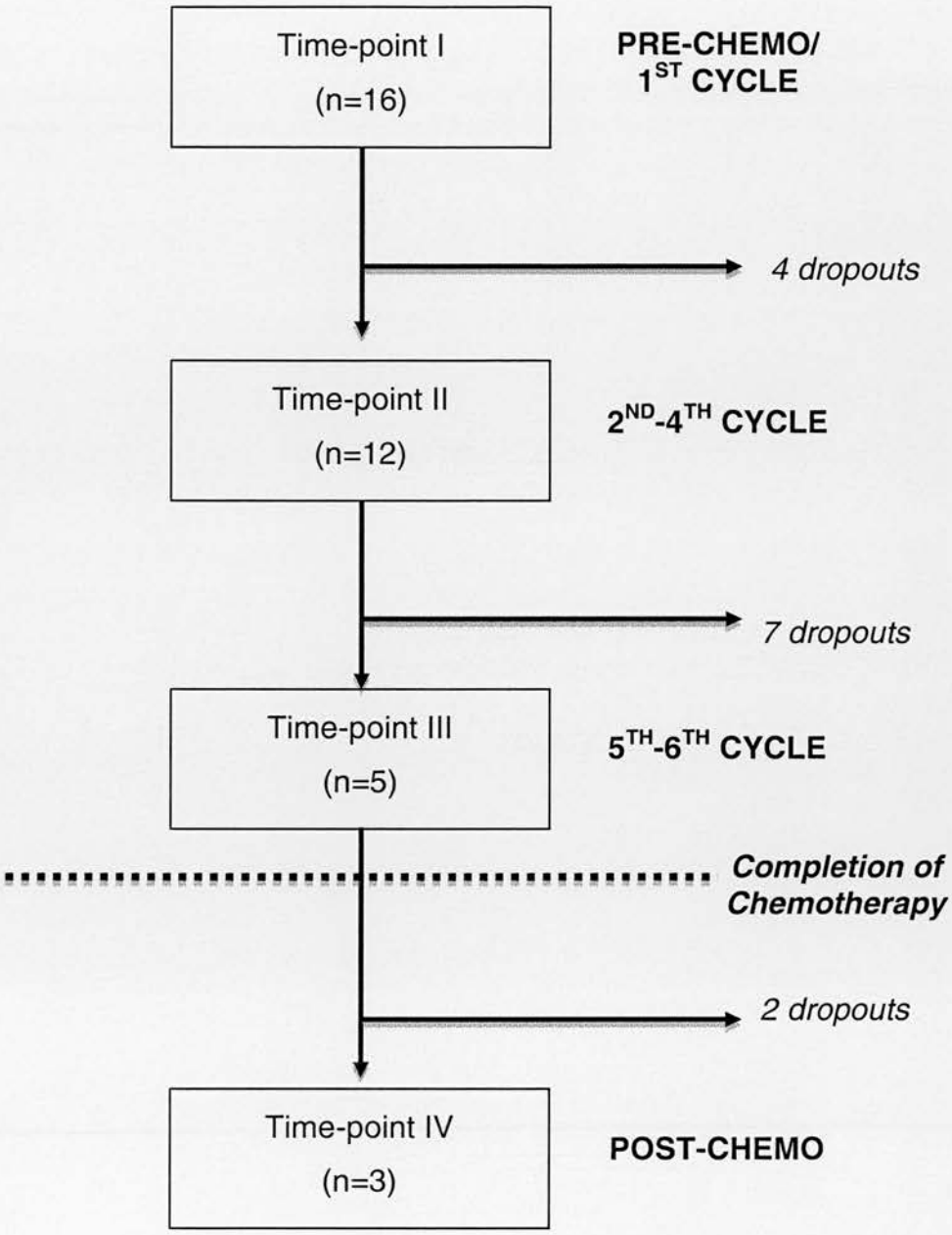

Figure 11.1 **Consort diagram of the recruited patients undergoing palliative chemotherapy (n=16).**

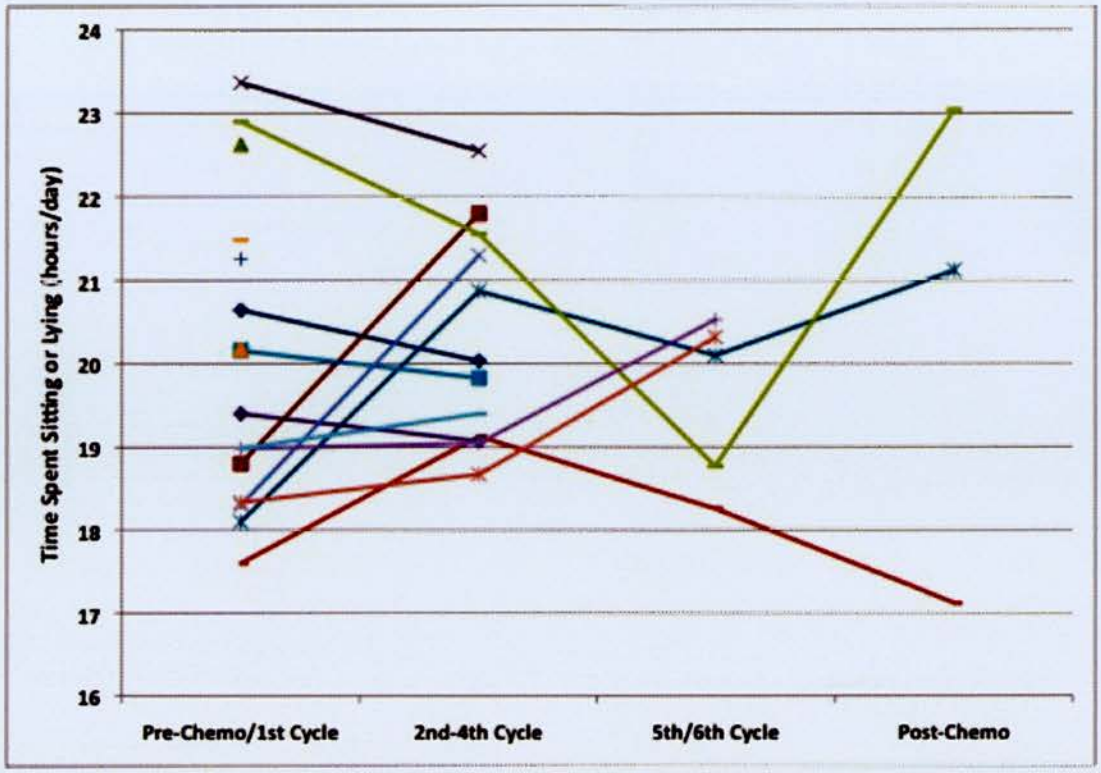

Figure 11.2A

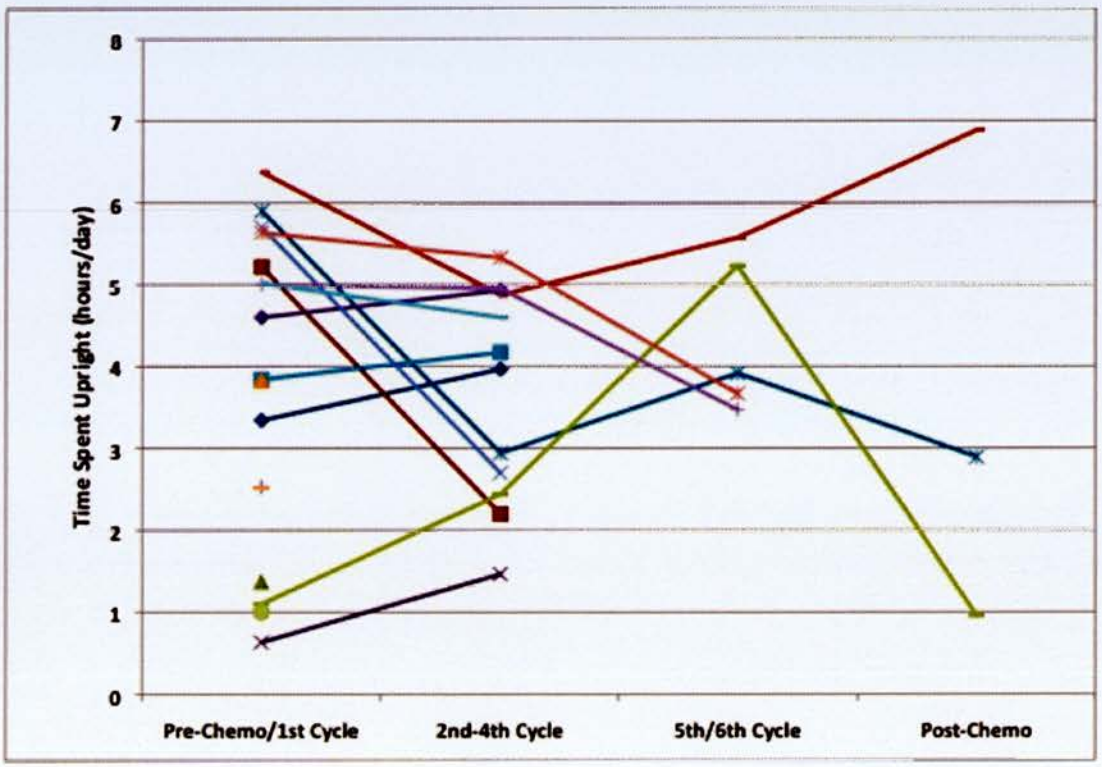

Figure 11.2B

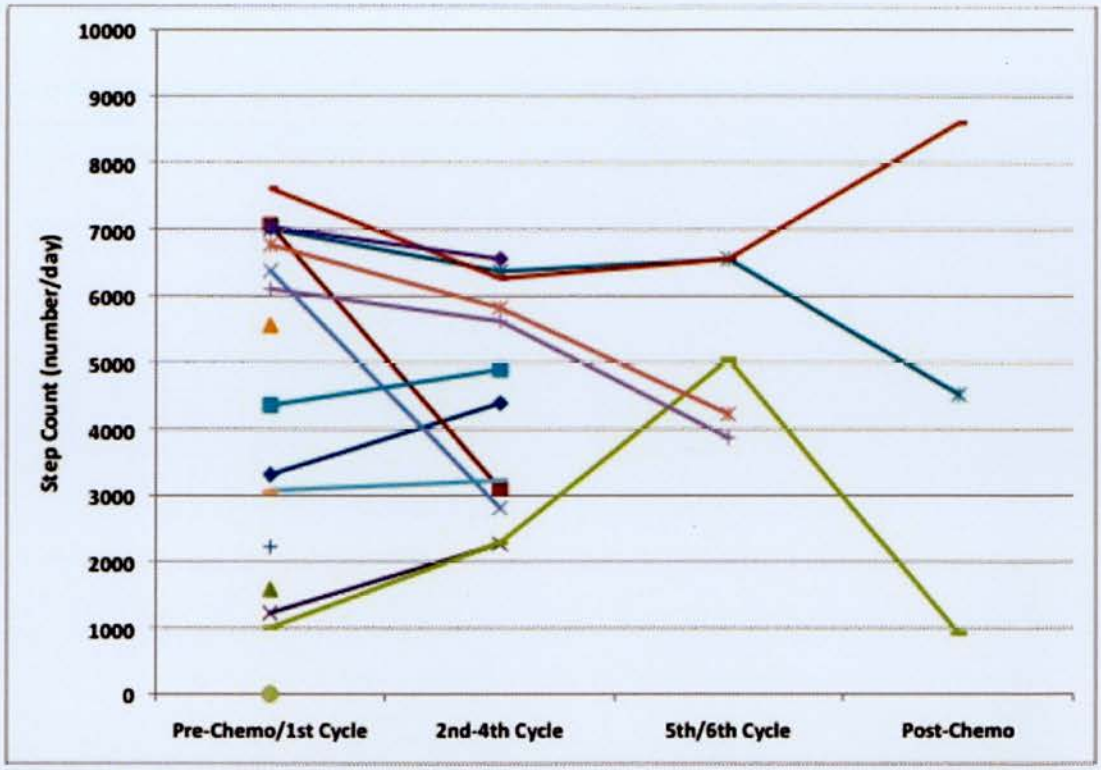

Figure 11.2C

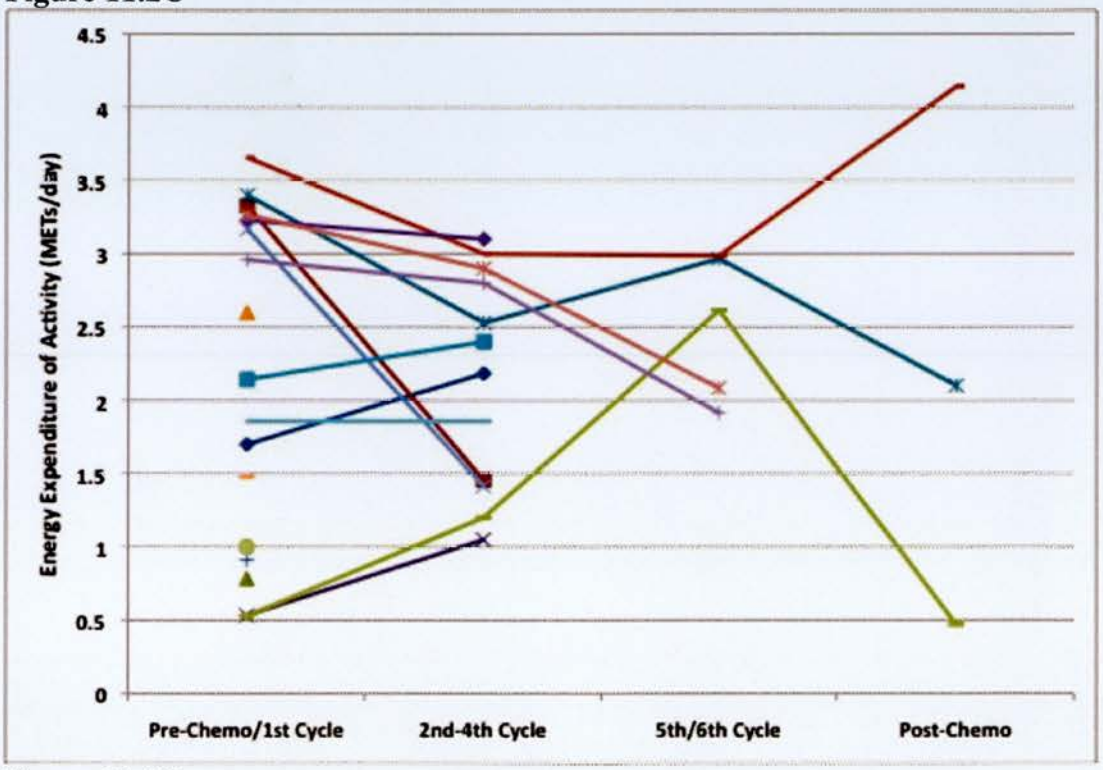

Figure 11.2D

Figure 11.2 **Repeated assessments of PA measures of individual patients during palliative chemotherapy. A) Time spent sitting/lying; B) Time spent upright; C) Step count; D) EEA.**

Patients demonstrated various PA responses following initiation of palliative chemotherapy, including increased PA, decreased PA and no change.

11.5.2.2 Longitudinal, related-samples, time-series analysis of patient groups

In those patients who underwent sequential assessment at time-points I and II (n=12), there were significant reductions in weight (median 72.5kg, range 50.6-128.8 versus median 68.5kg, range 46.1-132.2; p=0.038), AMC (median 25.3cm, range 17.8-32.0 versus median 23.8cm, range 18.1-28.7; p=0.034), and body fat (median 19.6kg, range 14.2-46.3 versus median 15.9kg, range 10.0-46.1; p=0.008) at time-point II compared with time-point I (WT for all comparisons), although there was no significant difference in LBM. These changes in body composition occurred despite the FAACT anorexia-cachexia syndrome (ACS) subscale score actually improving (p=0.021, WT). There were no significant changes in either the FAACT-TOI or FACIT-F TOI. Median PA measures also deteriorated between the two time-points but these changes did not achieve statistical significance due to the wide ranges of each measure (Figures 11.3a-d).

In those patients who underwent sequential assessments at time-points I, II and III (n=5), similar deteriorations in median PA measures were observed across the time-points, but again these changes did not achieve statistical significance (Figures 11.4a-d). In these patients, the FAACT Physical Well-Being (PWB) subscale score decreased significantly across the time-points (p=0.034, FT), although there was no change in the FAACT TOI or FACIT-F TOI. There were also no significant changes in nutritional status, implying that those patients who complete chemotherapy are those who do not develop severe cachexia. In contrast, those patients who experience nutritional depletion discontinue treatment early.

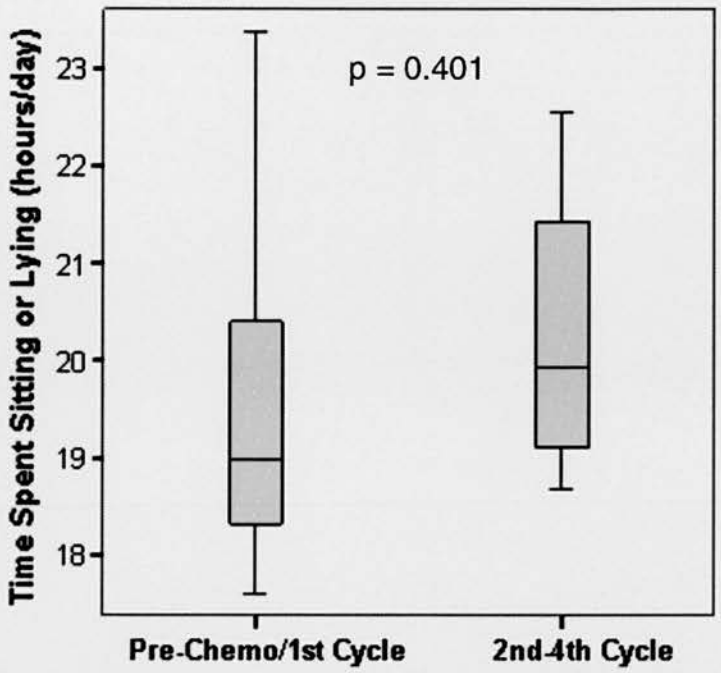

Figure 11.3A

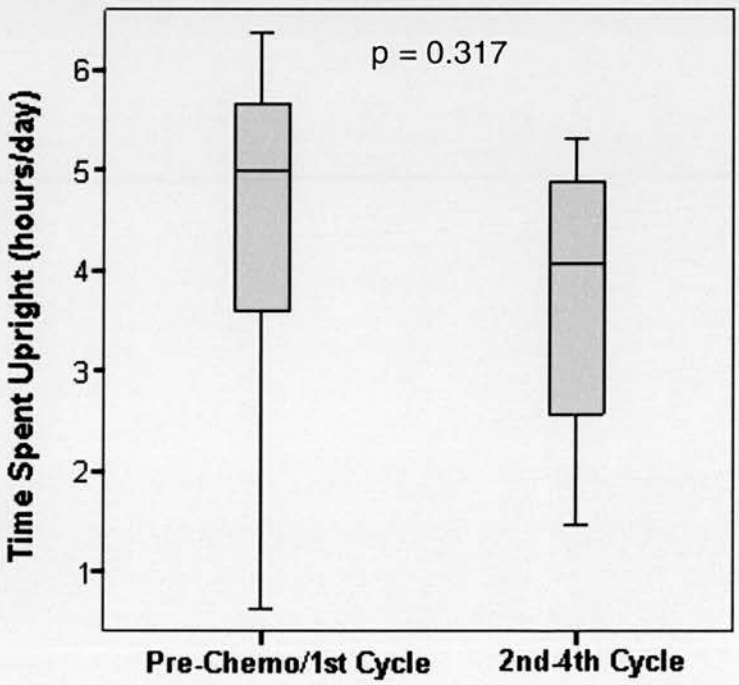

Figure 11.3B

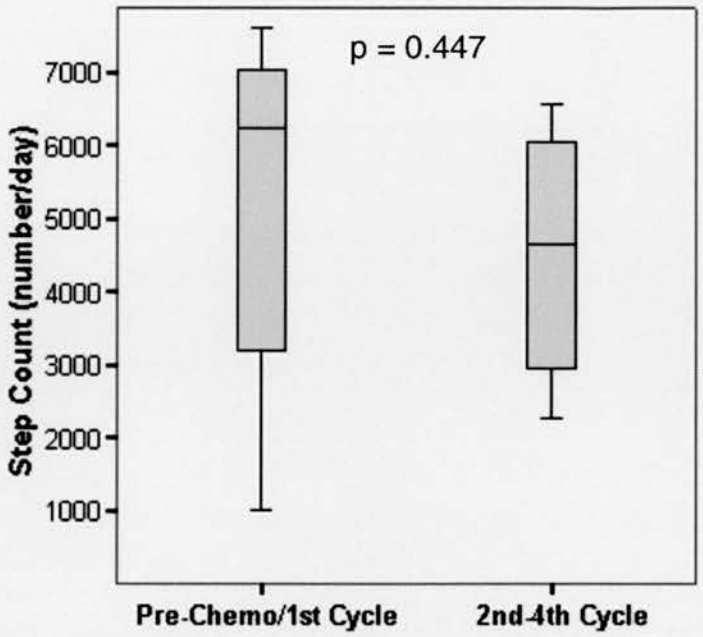

Figure 11.3C

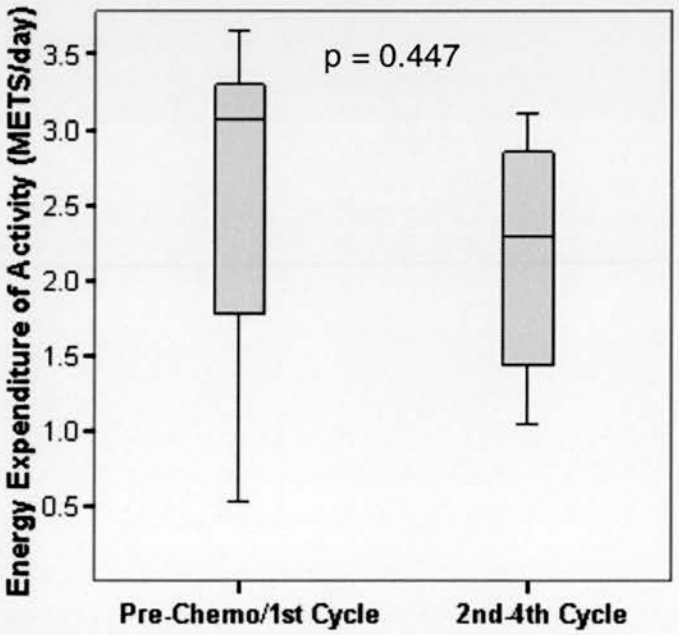

Figure 11.3D

Figure 11.3 **Box plots of different PA measures of patients undergoing sequential assessments (time-points I and II) during chemotherapy (n=12). A) Time spent sitting/lying; B) Time spent upright; C) Step count; D) EEA.** There was a non-significant trend (by WT) towards worsened PA as palliative chemotherapy progressed.

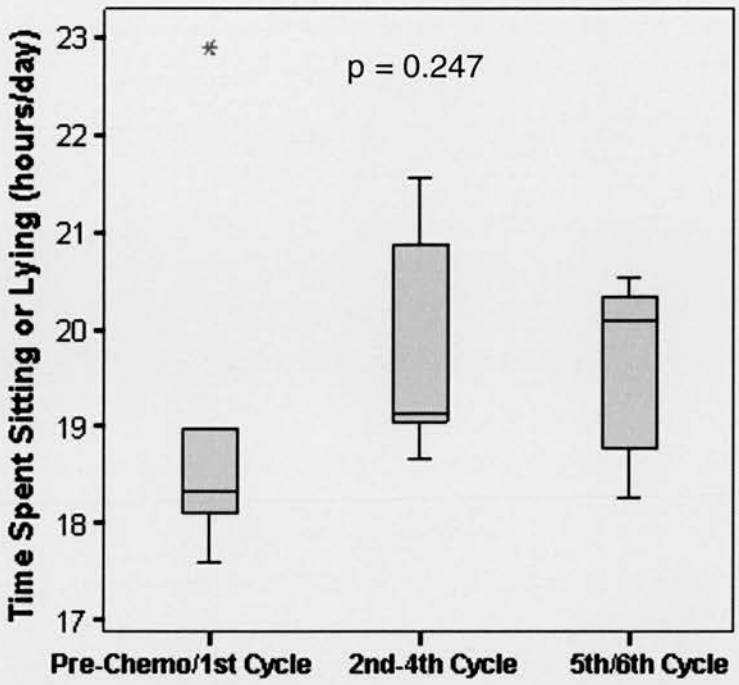

Figure 11.4A

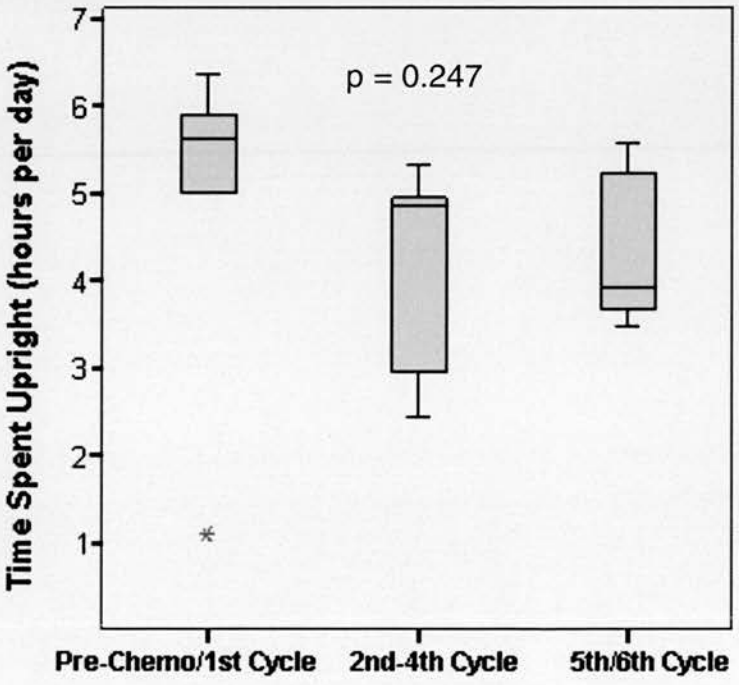

Figure 11.4B

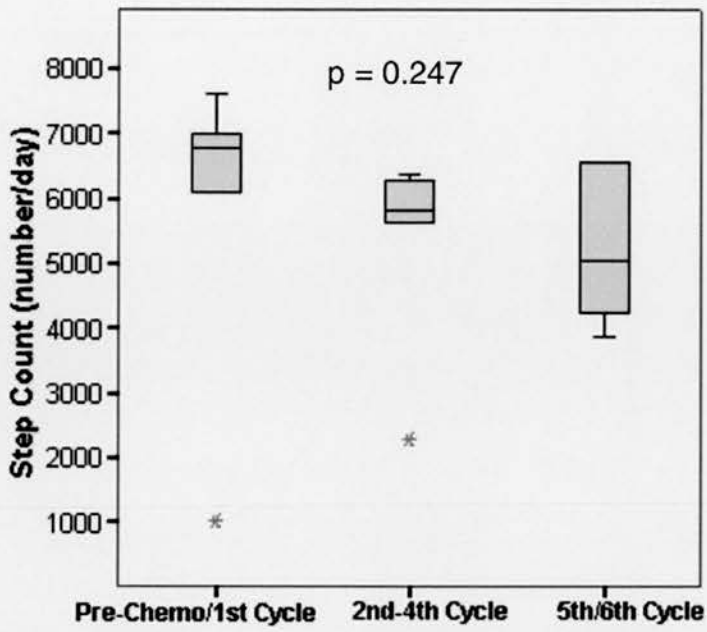

Figure 11.4C

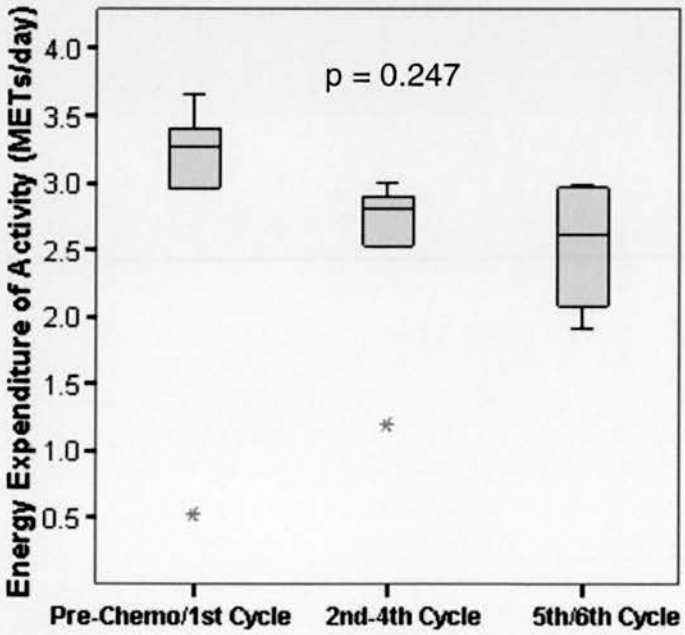

Figure 11.4D

Figure 11.4 **Box plots of different PA measures of patients undergoing sequential assessments (time-points I, II and III) during chemotherapy (n=5). A) Time spent sitting/lying; B) Time spent upright; C) Step count; D) EEA.** There was a non-significant trend (by FT) towards worsened PA as palliative chemotherapy progressed. Asterisks represent extreme outliers.

11.5.2.3 Relationship between PA and cessation/completion of treatment

Baseline PA measures at time-point I did not correlate with the number of chemotherapy cycles completed by each patient. Furthermore, baseline PA measures did not differ significantly between patients who completed 6 cycles of chemotherapy and patients who discontinued treatment early. In contrast, the EORTC QLQ-C30 PF score at time-point I demonstrated a trend towards correlation with chemotherapy cycle number (r=0.49, p=0.062), and the FAACT Functional Well-Being (FWB) (p=0.007), FACIT-F Fatigue (p=0.022), EORTC QLQ-C30 Global Health Status/QoL (p=0.040) and PF (p=0.040) scores were improved significantly in patients who completed chemotherapy compared with those who did not. The FACIT-TOI at time-point I also demonstrated a trend towards improvement in patients who completed chemotherapy (p=0.075).

Reverse time-series analysis demonstrated that median PA measures improved as the time duration before cessation/completion of treatment lengthened (Figures 11.5a-d), but these changes did not achieve statistical significance when all time-points (1, 2 and 3) were considered. However, the FAACT PWB subscale score did increase significantly as the time duration before cessation/completion of treatment lengthened (p=0.024, KWT).

When time-points 1 and 3 were compared directly, time spent stepping increased significantly (p=0.050, MWT), and the time spent sitting or lying (p=0.091), time

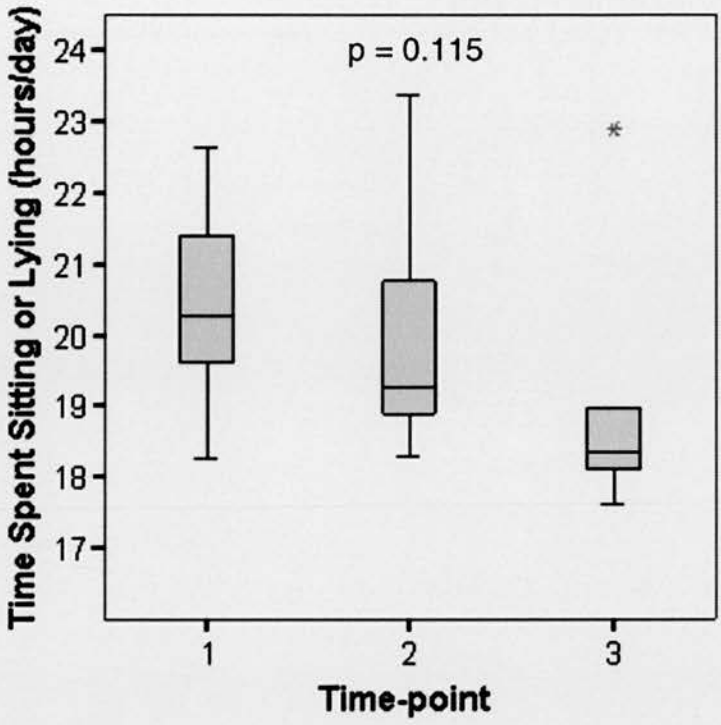

Figure 11.5A

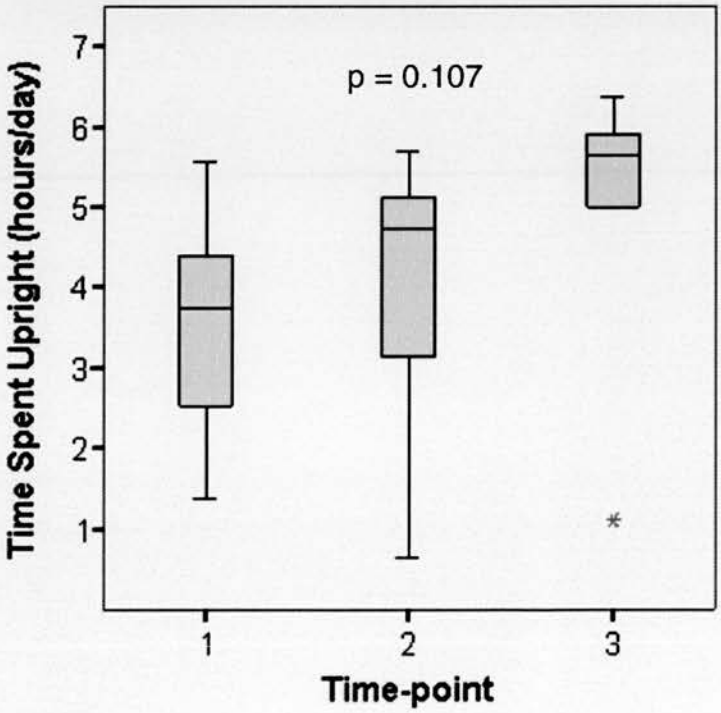

Figure 11.5B

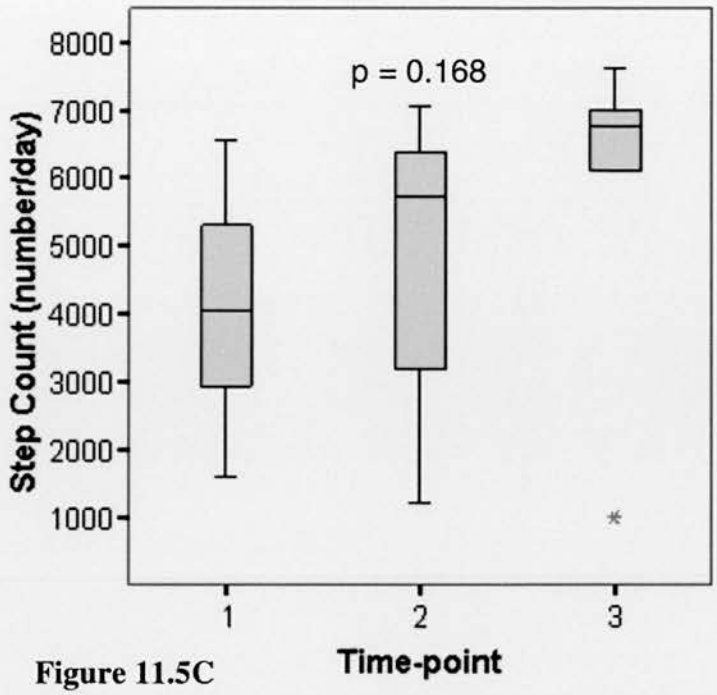

Figure 11.5C

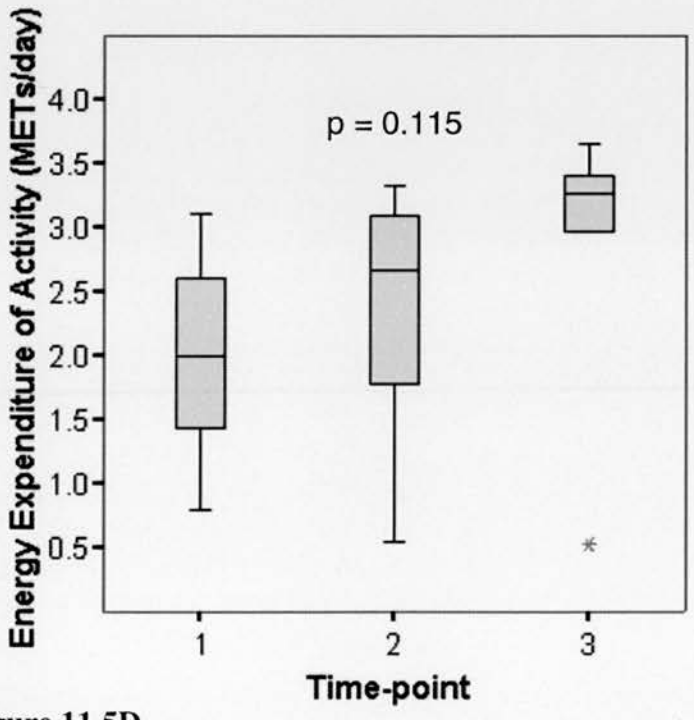

Figure 11.5D

Figure 11.5 **Box plots of reverse time-series analysis of different PA measures (n=16). A) Time spent sitting/lying; B) Time spent upright; C) Step count; D) EEA.**
Time-point 1 represents that closest to cessation/completion of chemotherapy. Time-points 2 and 3 are of increasing time duration before cessation of treatment. There was a non-significant trend (by KWT) towards improved PA as the time duration before treatment cessation increased. Asterisks represent extreme outliers.

spent upright (p=0.075), step count (p=0.091) and EEA (p=0.091) demonstrated trends toward PA improvement. Furthermore, the EORTC QLQ-C30 Fatigue score increased significantly between time-points 1 and 3 (p=0.033, MWT), and the Global Health Status/QoL (p=0.066) and PF (p=0.066) scores demonstrated trends toward increase.

11.5.3 Relationship between physical activity, nutritional status, quality of life and mood

Time spent sitting or lying (r=-0.50, p=0.003), time spent standing (r=0.54, p=0.001), time spent upright (r=0.50, p=0.003) and EEA (r=0.40, p=0.022) correlated with the FACIT-F TOI. Only time spent standing (r=0.36, p=0.043) correlated with the FAACT-TOI, despite the fact that time spent sitting or lying, time spent standing, and time spent upright all correlated significantly with the FAACT PWB (r values between 0.38-0.43; $p \leq 0.026$) and Functional Well-Being (FWB) (r values between 0.39-0.40; $p \leq 0.022$) subscale scores, individual components of the overall TOI. (EEA also correlated with the FWB subscale score (r=0.36, p=0.034)). The lack of further correlations between PA measures and the FAACT-TOI was due to the fact that PA measures did not correlate with the ACS subscale score, the third component of the TOI (along with PWB and FWB). However, despite the absence of an obvious relationship between PA measures and the ACS subscale score, time spent sitting or lying (r=0.35, p=0.047), time spent standing (r=-0.37, p=0.035), and time spent upright (r=-0.33, p=0.061) all correlated or demonstrated a trend towards correlation with percentage weight loss. LBM as a percentage of total body weight (rather than in kgs) correlated with time

spent upright (r=0.35, p=0.041) and correlated inversely with time spent sitting or lying (r=-0.35, p=0.041). LBM also correlated with time spent standing (r=0.33, p=0.057) and time spent stepping (r=0.310, p=0.075).

Regarding EORTC QLQ-C30 scores, all PA measures, with the exception of step cadence and up-down transition number, correlated with Global Health Status/QoL score with r values between 0.38-0.43 ($p \leq 0.027$), PF score with r values between 0.40-0.53 ($p \leq 0.007$), and Fatigue score with r values between 0.40-0.53 ($p \leq 0.020$). When considering mood, only step count correlated inversely with the HADS-Depression score (r=-0.34, p=0.044).

Percentage weight loss correlated inversely with the FAACT FWB (r=-0.47, p=0.007), FAACT-TOI (r=-0.52, p=0.004), FACIT-F TOI (r=-0.38, p=0.040), EORTC QLQ-C30 Global Health Status/QoL (r=-0.45, p=0.011), PF (r=-0.51, p=0.003) and HADS-Depression (r=0.47, p=0.006), demonstrating the negative impact that cachexia has on QoL and mood.

11.5.4 Relationship between physical activity and performance status

Excluding step cadence and up-down transitions, PA measures correlated with WHO and KPS performance scores with r values between 0.34-0.48 ($p \leq 0.041$). As an example of the relationship observed between PA and PS, Figure 11.6 shows a box-plot of step count across different scores of WHO PS. This figure demonstrates the wide range and overlap of objective levels of PA that can be described by a single PS score. Thus, patient assessments with WHO PS score 0 (n=10) had a

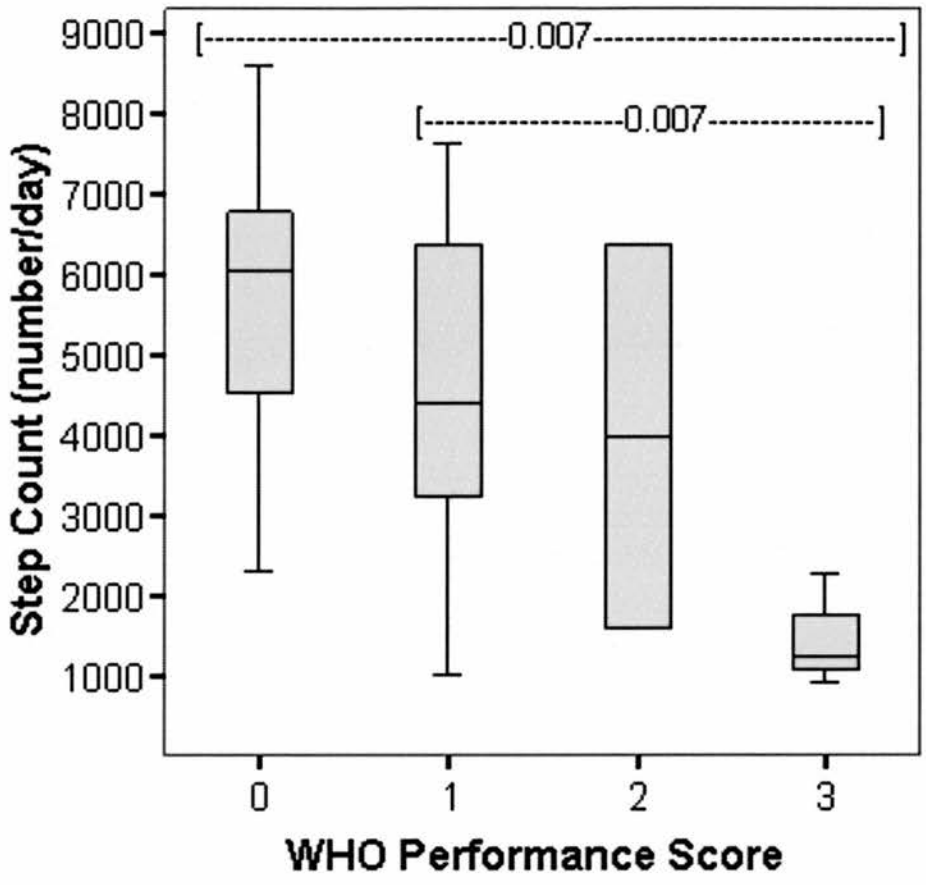

Figure 11.6 **Box plot of step count across different scores WHO performance status scores (n=36 assessments in total).**
Step count decreases as PS score increases (p=0.042, KW test). Results of MW tests comparing individual groups are shown on the figure.

median step count of 6046 steps/day (range 2293-8606); assessments with WHO PS score 1 (n=21) had a median step count of 4390 steps/day (range 1007-7619); assessments with WHO PS score 2 (n=2) had a median step count of 3985 steps/day (range 1588-6382); and assessments with WHO PS score 3 (n=3) had a median step count of 1228 steps/day (range 919-2277) (p=0.042, KWT).

11.6 Discussion

The present study describes a complex "journey" of PA taken by individual patients with advanced upper GI cancer as they receive palliative chemotherapy. Individual patients described worsened, improved or unchanged PA during treatment. Furthermore, in some patients, PA measures fluctuated at different time-points throughout treatment. However, the overall pattern (although largely statistically non-significant) was one of deterioration in PA during palliative chemotherapy. Similarly, when the data were analysed in reverse order from the point of cessation of chemotherapy, a reduction in PA was noted near to the time of treatment cessation. In particular, time spent stepping demonstrated a significant improvement as time duration before chemotherapy cessation lengthened. Larger longitudinal studies are required to confirm these findings and establish statistical significance. Furthermore, additional data on disease response to chemotherapy would help to tease out the separate impacts of chemotherapy and disease on PA.

Baseline FAACT FWB, FACIT-F Fatigue, EORTC QLQ-C30 Global Health Status/QoL and PF scores were able to predict chemotherapy completion, whereas PA measures were unable. It is not clear why this should be the case, but it may simply be a reflection of the wide variation of habitual levels of PA seen at the commencement of treatment. Surprisingly few data are available on the association between baseline QoL and chemotherapy completion rates [722], although it has been suggested that patients who have lower QoL scores may be less willing to continue therapy [498]. Many previous QoL studies have targeted patients undergoing follow-up after the cessation of therapy [513, 723]. However, baseline

QOL has been shown previously to be capable of predicting patient survival. The baseline EORTC QLQ-C30 PF scores predicted long-term survival in patients with metastatic colorectal cancer [724] whereas, in newly diagnosed patients with oesophageal cancer, an increase in the EORTC QLQ-C30 PF score of 10 points corresponded to a 12% reduction in the likelihood of death [725]. Further studies involving the pooling of various patient groups involved in activPAL™ studies are underway in order to analyse the effect of PA on cancer patient survival.

With the exception of step cadence and up-down transitions, PA measures correlated with EORTC QLQ-C30 Global Health Status/QoL, PF and Fatigue scores. These observations are an advance beyond previous smaller studies that could not demonstrate significant associations beyond PA and QoL [380]. Certain PA measures, namely time spent sitting or lying, time spent standing, time spent upright and EEA (but not step count), also correlated significantly with the FACIT-F TOI score. These findings confirm that many PA measures can be used as surrogate outcomes of QoL. The positive relationship observed between PA measures and various QoL scores is encouraging. Different QoL questionnaire tools are not indistinguishable and therefore cannot be assumed to be interchangeable [502]. However, if PA measures correlate with several different QoL tools, the objective assessment of PA could be considered a superior and overall index of QoL. Again, pooled studies of larger patient groups will characterise further existent relationships between PA measures and QoL.

Time spent standing correlated significantly with the FAACT-TOI score. The lack of a relationship between other PA measures and the FAACT-TOI was largely dependent on the fact that PA measures did not correlate with the FAACT ACS subscale score. At first glance, this observation appears difficult to explain as percentage weight loss correlated inversely with some PA measures and the ACS subscale score ($r=0.46$, $p=0.009$). However, it prompts the suggestion that PA measures do not adequately describe those symptoms identified by the ACS score. Certainly, of the 12 questions that compose the ACS subscale score, 10/12 pertain to patient appetite, early satiety, vomiting and general health, whereas only 2/12 questions relate to patient weight or body shape ("I am worried about my weight"; "I am concerned by how thin I look"). One might theorise that PA measures are more likely to be influenced by objective changes in body composition (e.g. visible reduction in weight) rather than subjective patient perceptions of decreased appetite. Thus, the emphasis of the ACS score on nutritional intake rather than body composition may be a contributory factor in the lack of association with PA measures. This supposition is supported by evidence that weight loss is a larger determinant (effect size 30%) than nutritional intake (effect size 20%) and chemotherapy (effect size 10%) of EORTC QLQ-C30 PF scores [726]. Nutritional intake was not formally assessed in the present study. However, percentage weight loss was recorded, and it was shown that patients had lost a median of 8.3% weight at baseline. Moreover, the institution of chemotherapy was associated with a further loss of weight of approximately 3.7% (4.0kg). The observed weight loss will be one mechanism that leads to the reduction of PA associated with chemotherapy, presumably through a reduction in functional LBM. However, it is worth noting

that, in the present study, BIA suggested that a large proportion of the lost body compartment was fat rather than skeletal muscle. This finding may explain why the observed reductions in PA associated with chemotherapy did not reach statistical significance. Larger reductions in LBM may be required before it is reflected by a significant impairment of patient PA. In the present study, LBM correlated with some PA measures, and this observation would be consistent with the above hypothesis.

Of all the PA measures, only step count correlated with depression scores. Further studies are therefore required to ascertain the relationship between mood and PA in advanced cancer. Interestingly, HADS-Depression scores also correlated significantly with weight loss, which implies that either mechanisms that work peripherally to produce weight loss also act centrally to cause depression (e.g. pro-inflammatory cytokines), or that undesirable changes in body composition affect patient mood adversely.

The present study demonstrated that worsening PS was reflected in lower levels of PA. However, it is apparent that subjective assessment of PS cannot be used to describe activities in detail. Furthermore, PS does not take into account a patient's objective, pre-morbid level of PA. Thus, two patients may have identical PS scores but one could be an elite athlete whereas the other leads a sedentary lifestyle. Therefore, PS scores offer a misleading impression of the actual level of PA or the finite deterioration of PA that has been caused by disease or subsequent treatment.

In the present study, patient assessments that had been assigned the same WHO PS score varied by as much as 6000 steps/day, or more than 5 times the lowest step count limit. The WHO scale differentiates PS scores of 2 and 3 on the basis of whether or not someone is 'up and about' more than 50% of the waking time. Assuming a waking time of 16 hours/day, not one assessment in the present study exhibited a measure of waking time spent upright of >50% (median percentage waking time spent upright 24.6%, range 4.0-43.0). If one considers the average day of a healthy but sedentary individual (e.g. office worker), one can see that even healthy individuals are unlikely to spend >50% of waking time upright, thus highlighting the artificiality and subjectivity of PS.

PA measures show promise as outcomes for use in trials of anti-cancer and anti-cachexia therapies, but their employment may now be timelier than ever. Recent studies have focused on the use of exercise as an anti-cachexia therapy in advanced cancer patients [727] [728] and PA measures would be the perfect outcomes to assess its efficacy and compliance. Most studies to date have been of breast cancer patients offered aerobic or resistance exercise programmes [418]. Initial results have been successful with median rates of uptake and completion of 63% and 87%, respectively [418]. A supervised high intensity exercise intervention in 269 cancer patients undergoing chemotherapy resulted in improvements in fatigue, PF, aerobic capacity (measured by maximal O2 consumption) and muscular strength, but not global QoL [729]. In lymphoma patients, aerobic exercise training resulted in improvement in PF, global QoL, fatigue, depression, cardiovascular fitness, and LBM [422].

In conclusion, palliative chemotherapy is likely to cause a deterioration in PA, although larger studies are required to confirm these findings and establish the predictive ability of PA measures on chemotherapy completion rates and survival. PA measures correlate with QoL scores, including global QoL and PF. Lastly, PA measures highlight the subjectivity and lack of detail offered by PS scores in advanced cancer. The objective assessment of PA is now a relatively simple procedure, and its use should be considered as a biomarker of skeletal muscle function in the development of outcome measures in trial methodology of anti-cancer and anti-cachexia therapies.

This chapter represents the last of the Results section. In the next section, the findings of the entire thesis are synthesised into a general discussion of the impact of this thesis on cancer cachexia research.

Part F – General Discussion, Bibliography and Appendices

Chapter 12 - General discussion

12.1 Summary of thesis findings and relevance to cancer cachexia therapy

At the end of each of the nine Results chapters of this thesis, there is a detailed discussion of the experimental findings. In this final Discussion, the work of the thesis is amalgamated, limitations are discussed and the results are placed in context. Furthermore, ongoing research studies based on the findings of this thesis are described.

In the management of cancer cachexia, there is a pressing need for biomarkers that are capable of recognising patients in the 'pre-cachectic' phase, prior to the development of significant weight loss or muscle wasting, because early multimodal intervention at this stage is more likely to be successful. These biomarkers should either function as mediators themselves, or reflect the activity of mediators, to identify both patients at risk and pharmacological targets. There is a further need for biomarkers that allow the assessment of response to treatment in key organs such as skeletal muscle. With these unmet clinical needs in mind, the general aims of the present thesis were to:

1. Investigate tumour-derived mediators of cancer cachexia (including MIC-1, a potential 'crossover' factor of both tumour and host derivation).
2. Investigate host-derived mediators of cancer cachexia.
3. Analyse metabolic/end-organ changes in skeletal muscle.

4. Investigate biochemical biomarkers of skeletal muscle wasting.
5. Investigate clinical biomarkers of skeletal muscle function.

Figure 12.1 represents a schematic diagram of the way in which each chapter of the present thesis has tackled a different component of cancer cachexia within the context of the above general aims. Upper GI cancer patients were chosen as the models for investigation in the clinical studies within this thesis as such patients are known to experience high rates of cachexia [4].

In Chapters 3 and 4, dermcidin mRNA expression, as a proxy of PIF expression, was analysed in human tumour tissue and immortalised cell lines. PIF is thought to be a possible tumour-derived mediator of skeletal muscle wasting in cancer cachexia, based on data derived predominantly from murine and *in vitro* models [17, 540]. However, controversy surrounds the existence of a potential human homologue of PIF (for a detailed analysis of the unresolved biology of PIF see Chapter 1.6.1, p.69). In particular, the mAb used to identify PIF has been reported as non-specific, and capable of binding to other common proteins such as albumin and Ig κ-light chains [208]. Moreover, clinical studies using this mAb do not associate PIF immunoreactivity specifically with malignancy, weight loss, muscle catabolism [208, 209] or survival [183, 208]. Other difficulties arise when considering that PIF appears to be transcribed from a gene (known as either *DCD* or *DSEP*) that, depending on both the extent of transcription and post-transcriptional/translational modification, may produce other functional proteins [194, 195]. The unprocessed-110 amino acid protein product is known as dermcidin

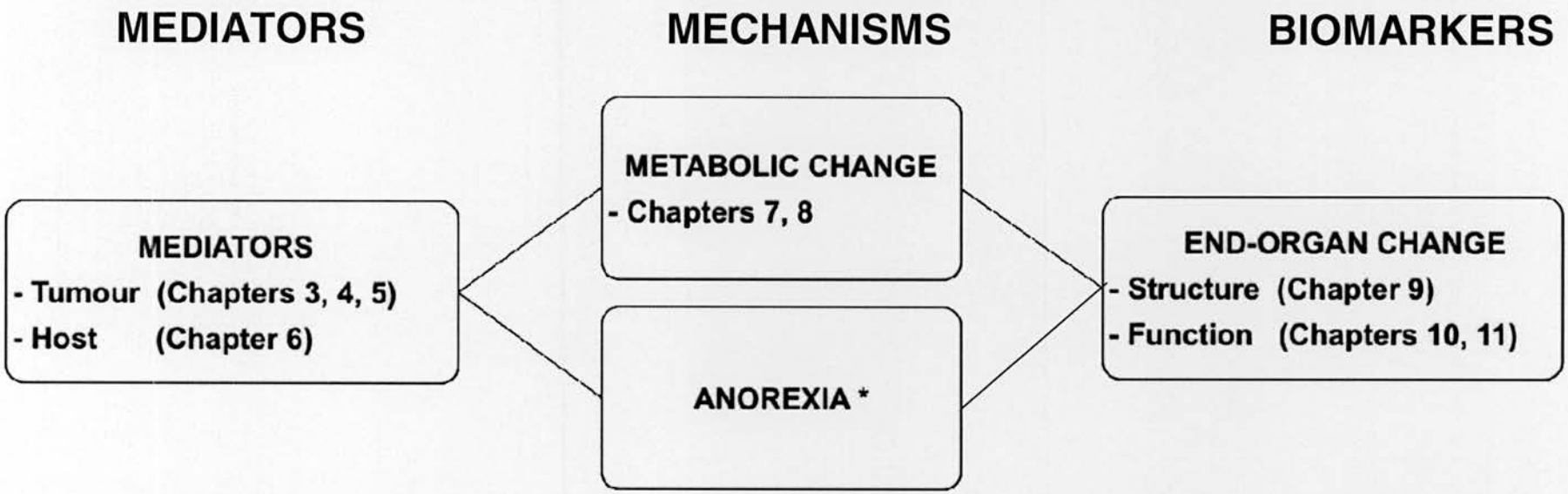

Figure 12.1 **Schematic diagram of the layout of the present thesis in regards to the unmet clinical needs within cancer cachexia.** The first section of the Results chapters (Part B) detailed investigations of cachectic mediators. Chapters 3 and 4 investigated the putative human homologue of PIF whereas Chapter 5 studied the tumour-derived mediator, MIC-1. Host-derived mediators, namely sex steroids and gonadotrophins, were investigated in Chapter 6. Mechanisms of skeletal muscle wasting were then investigated in Part C. Signs of metabolic change within skeletal muscle were demonstrated, namely DGC deregulation (Chapter 7) and PKR/eIF2α phosphorylation (Chapter 8). In Parts D and E, biomarkers of skeletal muscle wasting and function were investigated. Structural muscle end-organ damage was confirmed by the presence of breakdown products in cachectic urine in Chapter 9. In Chapters 10 and 11, the objective assessment of physical activity was studied as a potential biomarker of muscle function.
* Anorexia was not investigated formally or in detail but MIC-1 has been proposed as an anorectic agent in murine studies.

or HCAP, but this product incorporates several different sequence components, including the 30-amino acid PIF-CP (Y-P30), the 47–amino acid antimicrobial peptide found in human sweat (DCD-1), a 13-amino acid propeptide, and a 19-amino acid signal peptide (see Figure 1.6, p.72). Due to the lack of a reliable and sensitive method of PIF protein detection in human tissues/samples, dermcidin mRNA expression was used as a proxy of PIF expression within the present thesis. However, although dermcidin mRNA expression has been used previously as a human homologue of PIF during investigations of human cancer tissue [197], at present, there is no strong evidence that human PIF is indeed a downstream product of dermcidin (through an unknown mechanism of post-trasncriptional/translational processing), or that increased expression of dermcidin correlates with increased expression of human PIF.

Using real-time PCR, dermcidin mRNA was expressed in 60% of pancreatic cancer biopsies and 4% of OGC biopsies, but not in prostate cancer samples, demonstrating the variable expression of this gene in different cancer types. However, the finding that dermcidin was not expressed in primary prostate cancer was discordant with that of a previous study by Wang *et al* [197]. These differences may be due to either differing experimental techniques, including primer disparities (note the difference in findings between the *PIF* and *HCAP* primers in Chapter 4) or differences in the nature of the positive control. However, the low expression of dermcidin mRNA in the majority of non-prostate cancer samples would support the conclusion that dermcidin is unlikely to play a significant pro-cachectic role in primary prostate cancer.

Wang *et al* also identified dermcidin expression in metastatic prostate cancer deposits [197], and thus it could be hypothesised that high levels of dermcidin expression by tumour cells may only take place in the context of advanced metastatic disease. However, although distant metastatic deposits were not included in the present thesis, immortalised cell lines with a metastatic origin were analysed (e.g. PC-3M, which was originally derived from liver metastases following injection of human prostate adenocarcinoma cells into nude mice). Dermcidin mRNA expression was absent in such cell lines using real-time PCR (PC-3M has a C_T value of 40). In fact, PC-3M cells required transfection with the pcDNA3.1+DCD plasmid (during the generation of the positive control) to ensure high expression levels of dermcidin mRNA (C_T value of 23.1). Ultimately, the question of dermcidin expression by metastatic deposits would be best answered by a matched-pair comparison of expression levels between primary cancer tissue and metastases in patients with advanced malignancy. However, currently, the findings of the present thesis do not support or suggest increased dermcidin expression in metastatic prostate cancer at least.

It is interesting that dermcidin mRNA was qualitatively detectable by RT-PCR in several pancreatic and prostatic cell lines (including CFPAC, MIA-Pa-Ca-2, PC-3, PC-3M, and DU145), but that quantitative expression by real-time PCR demonstrated that the finite levels of expressed dermcidin mRNA were either low or absent. This suggests that RT-PCR is capable of amplifying tiny amounts of dermcidin mRNA sufficiently to allow detection by gel electrophoresis, despite the fact that actual expressed levels of mRNA (and hence protein) may be insignificant

clinically. (Four i.v. injections of 7-10μg of murine PIF induced weight loss in mice, even though some of the compound weight could be attributed to albumin bound to PIF [185, 730]. Moreover, murine PIF is reported as having high affinity for its receptor [211]. However, the finite minimal concentration of a human homologue of PIF that is required to have biological action *in vivo* is unknown). Thus, real-time PCR may be more useful (than RT-PCR) in future studies as a true measure of dermcidin mRNA activity.

Sequence analysis of the amplified dermcidin RT-PCR products derived from cell lines did not detect any sequences that would increase the probability of *N*- or *O*-glycosylation and thus increase the likelihood of a functional human PIF [203, 204]. The primers used for this analysis yielded variable results. *PIF* primers amplified dermcidin cDNA from 8/10 cell lines, whereas *HCAP* primers amplified dermcidin from only 6/10 cell lines and were not successful at permitting sequence identification. Clearly, the choice of molecular analytical tool can affect the results obtained when investigating such a controversial gene as *DCD*.

In summary, the failure of the present thesis to identify robust expression of dermcidin in human cancer tissue and cell lines, in the context of no accurate methodology to detect PIF protein, means that further investigation of a putative human homologue of PIF remains difficult at this time. Large clinical studies of dermcidin expression within patient tumour samples are unlikely to demonstrate success of dermcidin as an early, sensitive biomarker of muscle wasting, as expression is likely to be low or absent in many tumours. If undertaken, such studies

should focus predominantly on cancer types where there is evidence (albeit small) of regular dermcidin expression (e.g. pancreatic cancer). Initially, smaller studies that utilise novel PIF protein detection methods (e.g. protein capture using the putative PIF receptor) may be more useful to firstly confirm the presence of human PIF, and then secondly, validate a sensitive measure of detection. Once done, these studies could then be expanded within larger patient populations to assess the usefulness of PIF as a biomarker of human cancer cachexia.

In Chapter 5, plasma MIC-1 was investigated within a cohort of patients with OGC (n=293) as another potential tumour-derived mediator in the aetiology of cancer cachexia [254]. Initially, MIC-1 had been selected for investigation as a potential mediator derived from both tumour cells and TAMs i.e. a factor that crosses the boundary between tumour-derived and circulating mediators. However, although marcrophage-derived secretion of MIC-1 has not been analysed formally within the context of cancer, previous data obtained from human, murine and *in vitro* studies of prostate cancer has demonstrated that MIC-1 appears to be derived from tumour cells [253, 254]. Assuming that the same regulatory mechanisms of MIC-1 expression exist in OGC (the present thesis did not investigate the cellular source of MIC-1), MIC-1 could also be considered a tumour-derived mediator within this cancer type. Certainly, plasma MIC-1 concentrations were associated with tumour site, stage and grade in OGC patients, supporting the hypothesis that OGC cancer cells influence MIC-1 expression. However, plasma MIC-1 concentrations also correlated independently with markers of systemic inflammation (namely CRP and the mGPS), implying that MIC-1 acts as either an upstream inducer of the APPR or,

possibly, a downstream effector (although this latter hypothesis would require reverse crosstalk between the liver and the tumour, a potential pathway that has not yet been investigated in cancer cachexia). On linear regression, the effect size of plasma MIC-1 on the variation observed in plasma CRP was only small (5.9%). However, the association between plasma MIC-1 and CRP was highly significant, implying that tumour cells are not necessarily the only source of circulating MIC-1 and that other inflammatory cells (e.g. monocytic PBMCs and macrophages) might also secrete additional MIC-1. Thus, in future studies of factors that may induce the APPR in cancer cachexia, MIC-1 would be a suitable candidate for investigation. Co-culture experiments using PBMCs/macrophages, isolated human hepatocytes and anti-MIC-1 antibodies may be useful to confirm both the cellular source of MIC-1 secretion in cancer patients, and the end-organ effects of MIC-1. Figure 12.2 represents a diagram of the hypothesised role of MIC-1 in the aetiology of the hepatic APPR in cancer cachexia.

Studies in mice with xenografted prostate tumours have suggested that the mechanism underlying MIC-1-induced weight loss is anorexia caused by reduced hypothalamic NPY expression and increased POMC expression [254]. However, in patients with OGC, plasma MIC-1 concentrations did not correlate independently with measures of nutritional status. Furthermore, plasma MIC-1 correlated only weakly with diet score. Taken together, these observations imply that the central modulation of appetite control observed in murine studies of MIC-1 may not hold true in humans, particularly those with OGC. This assumption could be tested using clinical studies that measure accurately dietary intake and/or employ functional MRI

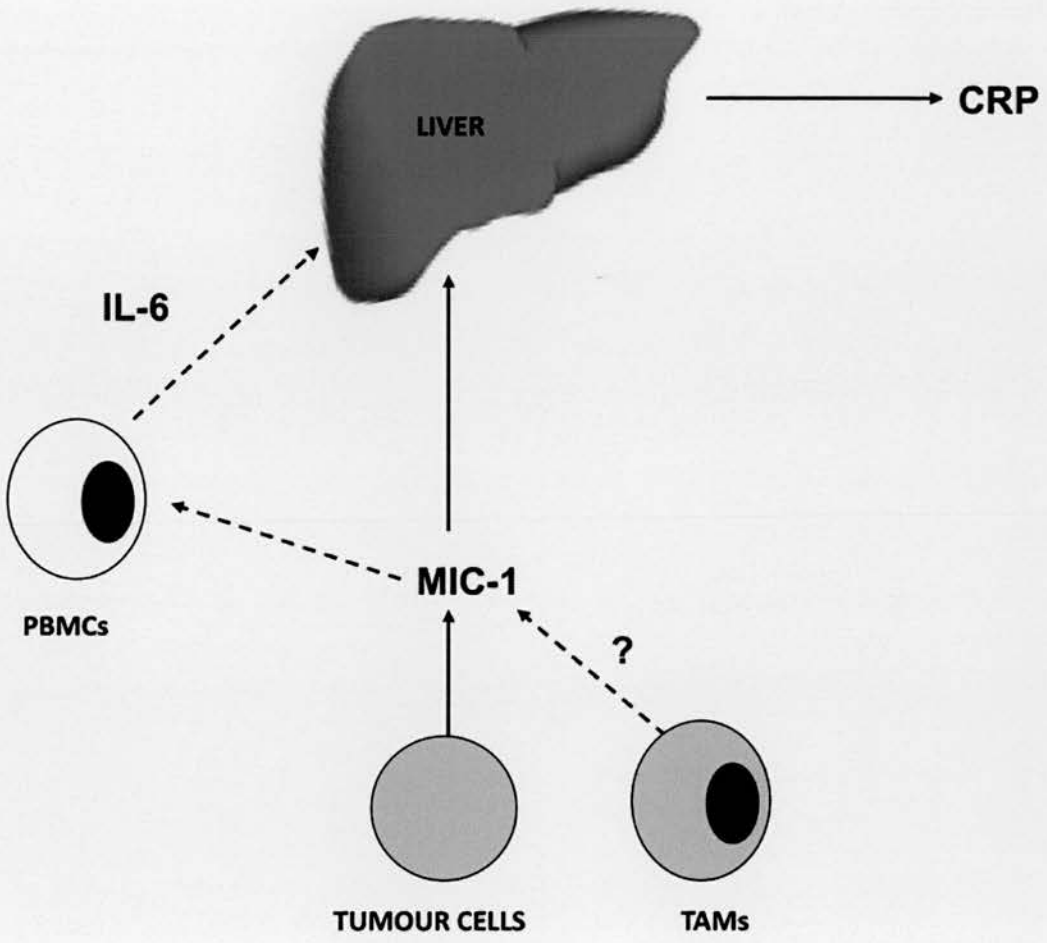

Figure 12.2 **The hypothesised role of MIC-1 in the aetiology of the hepatic acute phase protein response in cancer cachexia.**
MIC-1 is secreted by tumour cells to initiate a small but significant induction of the acute phase response, including a rise in plasma levels of CRP. At the present time, it is unknown if TAMs are involved in the secretion of MIC-1, or if MIC-1 acts directly on the liver or works indirectly through activation of PBMCs with further release of other inflammatory intermediates such as IL-6. Dotted lines represent possible, as yet uninvestigated, pathways.
CRP = C-reactive protein; IL-6 = interleukin 6; MIC-1 = macrophage inhibitory cytokine-1; PBMC = peripheral blood mononuclear cell; TAM = tumour-associated macrophage.

of the brain following administration of exogenous MIC-1. As there was no independent association between MIC-1 and nutritional status, and the effect size of MIC-1 on CRP was small, it is perhaps not surprising also that plasma MIC-1 was not an independent determinant of survival on multivariate analysis.

In summary, the present thesis has shown a potential role for MIC-1 in the induction of the APPR in humans with cancer cachexia, but not nutritional depletion. Therefore, it would appear unlikely that plasma MIC-1 concentration could function as a sensitive, early biomarker of muscle wasting. In any future effort to establish MIC-1 as a biomarker, a final consensus would need to be drawn regarding the true normal range of plasma MIC-1, and whether or not this varies with age (as suggested by the present thesis). At this present time, in the face of a lack of human data linking MIC-1 to nutritional status, it would be difficult to justify randomised controlled trials of targeted anti-MIC-1 therapies as a means of ameliorating cancer cachexia.

In Chapter 6, the topic of focus changed from tumour-derived mediators to host-derived circulating mediators. In particular, the potential role of neuroendocrine (sex steroid) alterations in the aetiology of cancer cachexia was investigated. Sex steroid abnormalities were hypothesised to be involved in cancer cachexia because of their roles in normal muscle homeostasis [731]. In adult males, the maintenance of muscle mass is controlled, in part, by testosterone acting through the AR and via other indirect mechanisms (see Chapter 1.5.1.1, p.56), whereas, in elderly females, post-menopausal loss of oestradiol is considered to be one of the main causes of

age-related sarcopenia [611]. For the present study, a cohort of advanced pancreatic cancer patients (n=167) was chosen as the model of investigation as pancreatic cancer is associated with the most aggressive form of cachexia and its routine treatment does not involve anti-androgen therapy (c.f. prostate cancer). In male patients, hypogonadism (defined as a sub-normal level of cFT) was found in 73% of male cancer patients. Futhermore, hypogonadism (defined as sub-normal levels of either cFT or TT) was associated with worsened nutritional status, increased weight loss, systemic inflammation, and shortened survival. The reasons for the high prevalence of hypogonadism within male cancer patients is not known, but hypothesised reasons include testicular suppression by pro-inflammatory cytokines, and elevation in SHBG levels causing reduced levels of bioavailable testosterone (SHBG itself may be an acute phase reactant in males with cancer as elevated serum levels correlated with systemic inflammation [586]). Furthermore, one must consider that hypogonadism in elderly males with cancer is also occurring on a background rate of 20% [584] seen in otherwise 'healthy' males aged 60 to 80 years. Perhaps one way in which to consider hypogonadism in the context of cancer cachexia might be as a 'sick eugonadal syndrome' (analogous to the sick euthyroid syndrome). In this way, the hypothalamic-pituitary-gonadal axis is not dysfunctional or structurally abnormal but is responding to the presence of a non-gonadal illness (i.e. cancer cachexia) in an aberrant fashion. The mechanism by which this sick eugonadal syndrome links to worsened survival is unclear, however, as the limited longevity of advanced pancreatic cancer patients would not support hypogonadism-driven muscle wasting as the sole cause of death. Iatrogenic induction of hypogonadism in males with prostate cancer only causes loss of LBM at a rate of

2% per 6 months [600], and so it is unlikely that muscle effects alone would result in demise. Rather, it is likely that hypogonadism acts as an overall frailty index that reflects and incorporates several poor prognostic factors including, but not limited to, the presence of tumour with systemic effects, ongoing muscle atrophy and the presence of systemic inflammation.

Opioids inhibit the release of hypophyseal gonadotrophins and are thus an iatrogenic cause of hypogonadotrophic hypogonadism [587]. In the present thesis, opioids were also associated with worsened outcomes in male cancer patients. Within the context of the present thesis, it was not possible to determine if this association was the result of an opioid-induced anti-androgenic effect or a reflection of the fact that opioids are often administered to patients with progressive advanced disease. Previous studies have shown pain at diagnosis to be an independent poor prognostic factor in pancreatic cancer [582], suggesting that the host inflammatory response [613], pain-induced stress or the pattern of tumour invasion could also underlie any detrimental response to opioids.

Clearly, the present thesis was only an association study and so cause and effect does need to be establised by therapeutic intervention trials. However, taken together, the results would support trials of androgen replacement (e.g. SARMs) as an anti-cachexia agent in male cancer patients. To date, similar trials have been carried out in HIV patients [107] and aged men [108], whereas Phase I and II studies have been carried out in the cancer population [106]. Future trials could be stargeted specifically to those male cancer patients who have documented

hypogonadism. Such targeting of patients with documented hormonal abnormalities might prevent a lack of positive findings, which has hampered previous trials of hormonal therapies (e.g. flutamide [605], tamoxifen [607]) in patients with pancreatic cancer. Additional study arms could be included that combine androgen replacement with anti-inflammatory therapies, particularly as a recently published study has suggested that hypogonadism and systemic inflammation can be considered as additive, synergistic problems, which, if both present, place the patient in a very poor prognostic group [732]. In the present thesis, it has been shown that hypogonadism and systemic inflammation are related intimately, and thus it may not be possible to separate fully the two conditions (Figure 12.3 represents a proposed mechanism for the whole interaction between sex steroids, systemic inflammation and opioid therapy). However, a synergistic approach to treatment with combination anti-androgen/anti-inflammatory may be superior to single therapy strategies. In any future trial, male patients receiving opioids should also be considered as high risk of becoming hypogonadal, and, where possible, alternative methods of analgesia (e.g. coeliac axis blockade) could be explored. However, it is not ethical to withdraw opioids from a patient in significant pain if there is no suitable analgesic alternative, and such a strategy should not be supported.

Pancreas is a sex steroid-responsive tissue [601-603] and therefore pancreatic cancer has been the subject of a number of experimental hormonal therapies. At first glance, one might expect that previous trials of flutamide, an anti-androgen, should have resulted in worsened survival of pancreatic cancer patients, rather than the lack

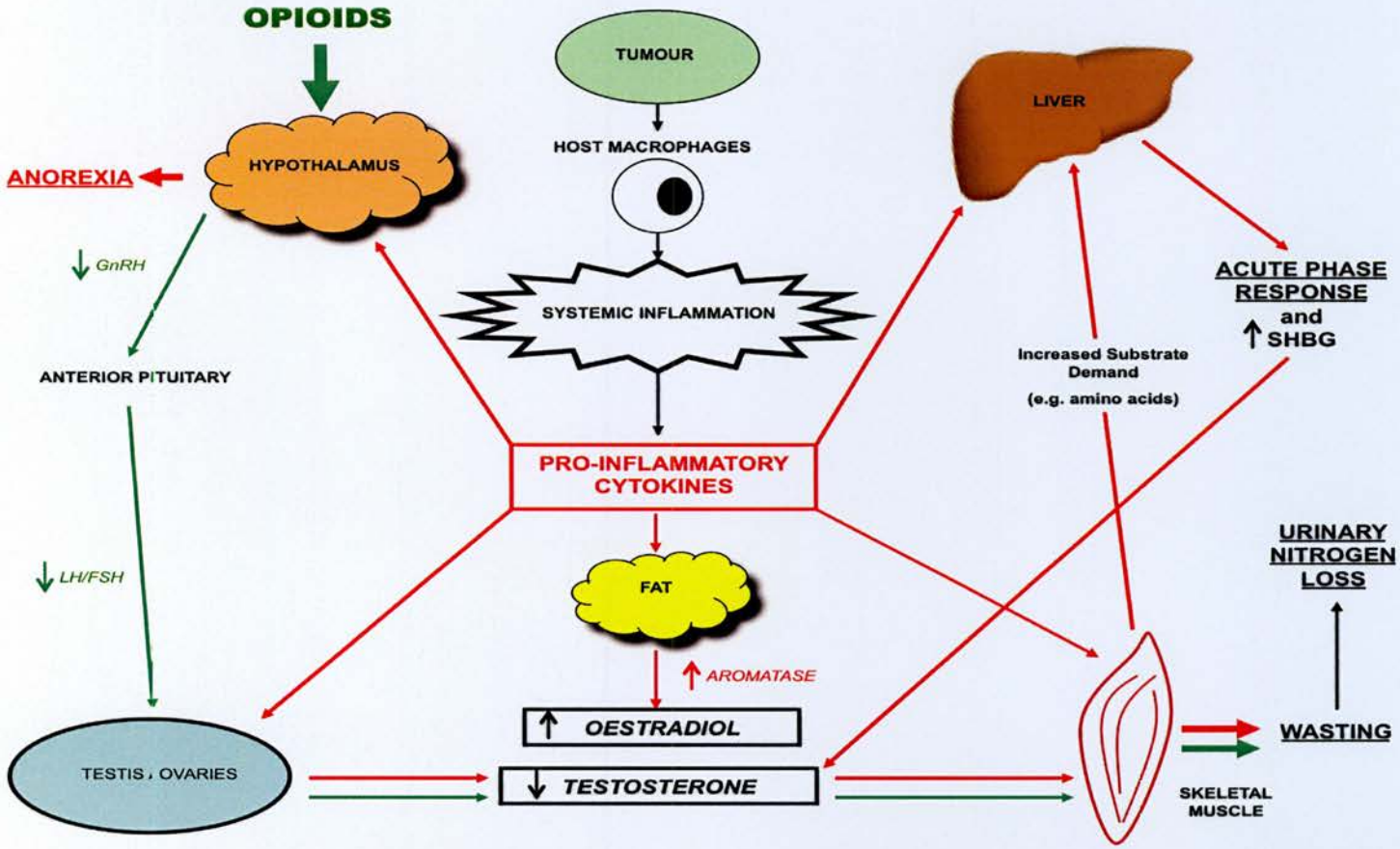

Figure 12.3 **Proposed cachectic mechanisms in the interaction between pro-inflammatory cytokines and the hypothalamo-pituitary-gonadal axis within cancer cachexia.**
Red pathways depict pathways associated with systemic inflammation whereas green pathways depict opioid-induced pathways. Pro-inflammatory cytokines act on the testicles to suppress testosterone in males, whereas, in females, cytokines stimulate peripheral aromatase activity to increase oestradiol production. Opioids induce hypogonadotrophic hypogonadism in males through central mechanisms.

of positive findings observed in some studies [605]. However, such trials have been carried out in only small mixed populations of both men and women.

Although hypogonadism was the main finding in male patients with advanced pancreatic cancer, relative 'hyperoestrogenism' was the chief observation in females. Eighteen percent of elderly female pancreatic cancer patients exhibited premenopausal serum concentrations of oestradiol. Furthermore, such hyperoestrogenism was associated with systemic inflammation and worsened survival, but not depleted nutritional status. Thus, although androgen replacement in male patients might be a suitable strategy with which to improve nutritional status and possibly survival, anti-oestrogen therapy in female patients might extend survival without impact on nutritional or functional status. The mechanism underlying the aetiology of hyperoestrogenism was hypothesised as cytokine-induced stimulation of extragonadal aromatase activity, but the mechanism underlying the deleterious effects of hyperoestrogenism remains unclear. At the very least, hyperoestrogenism is a marker of systemic inflammation and therefore one might expect it to correlate with shortened survival.

Female hyperoestrogenism was less prevalent than male hypogonadism (18% of female patients versus 73% of male patients), and thus a targeted strategy of hormonal manipulation might be even more important in female patients to avoid negative findings. Previous trials of anti-oestrogen therapy (tamoxifen) in mixed populations of men and women with pancreatic cancer have yielded variable results, although several studies have shown an improvement in survival [608, 610].

However, the relatively low prevalence of female hyperoestrogenism might imply that the beneficial results of tamoxifen observed in all-comer trials must represent a tumour effect rather than a body-wide effect of hormonal manipulation. In any future trials, augmentation of hormonal therapies with anti-inflammatory agents would appear to be as valid in female patients as in males.

One limitation of the current thesis is that serum hormone levels were only assessed at a single time point. It would be interesting to observe the natural history of sex steroid concentrations over the entire time course of a cancer patient's disease and to correlate these concentrations with markers of the APPR and measures of nutritional status. It is known that systemic inflammation fluctuates in severity with time, and thus, it would be expected that sex steroid concentrations might mirror such inflammatory variations. However, in the hypothesised 'sick eugonadal syndrome' model, one would anticipate that, as the underlying disease progresses and the severity of cachexia increases, abnormalities in the hypothalamic-pituitary-gonadal axis might worsen. Derangement of the neuroendocrine axis system might even represent a key mechanism in determining the mode of death in pancreatic cancer, as low testosterone levels in males and high oestradiol levels in females might confer increased susceptibility to cardiac arrhythmias [733]. A longitudinal, observational study of sex steroid levels in cancer might also provide clues as to the best timing of administration for hormonal therapies.

In summary, male hypogonadism would appear to represent a potential marker of cachexia to be employed in future studies, whereas female hyperoestrogenism,

although not necessarily a marker of depleted nutritional status, is also a marker of poor patient prognosis. These results demonstrate sexual dimorphism between men and women regarding the predominant hormonal patterns in advanced pancreatic cancer. This opens the field to the possibility of other sex-specific alterations in cancer cachexia. It has been known for several years that male patients with lung cancer experience an eightfold faster rate of weight loss and shortened survival compared with female patients matched for disease stage [734]. Furthermore, in a recent CT analysis of body composition, 61% of male patients with NSCLC were classified as sarcopenic compared with only 31% of women [29]. Moreover, recent reviews of the literature have suggested that sexual dimorphism influences the regulation of muscle mass in response to ageing. In particular, younger women are especially responsive to training compared with older women and men of all ages, whereas postmenopausal women exhibit a greater anabolic resistance than older men [735, 736]. This sexual dimorphism may also extend to forms of cachexia caused by chronic diseases other than cancer. For example, neutron activation analysis of patients with liver cirrhosis demonstrated that protein depletion was significantly more prevalent in men (63%) than in women (28%) [737]. Further studies of other disease types are awaited.

Chapter 6 was the last to consider the role of mediators (be they tumour- or host-derived) in the aetiology of cancer cachexia. In Chapters 7 and 8, metabolic changes within skeletal muscle as a result of mediator action were investigated. In Chapter 7, the prevalence of DGC deregulation within rectus abdominis samples from a cohort of patients with OGC (n=27) was analysed. The DGC is a membrane-

bound protein complex that connects the ECM with the intracellular cytoskeleton, and protects skeletal muscle from contraction-induced injuries [317]. Genetic abnormalities in this complex are known to be the cause of certain types of muscular dystrophy [631], but the possibility that acquired DGC abnormalities might relate to cancer cachexia was explored within the present thesis. The presence of DGC deregulation, incorporating reduced dystrophin expression, hyperglycosylation of β-DG and hyperglycosylation of β-SG, was found in 63% of patient samples. Furthermore, in the same samples, reductions in DGC complex association and actin-myosin complex association were also seen. DGC deregulation was associated strongly with the presence of systemic inflammation, suggesting that circulating pro-inflammatory cytokines may be the causative mediators that induce sarcolemmal structural abnormalities. Furthermore, DGC deregulation was associated with a trend towards worsened performance status, implying that DGC deregulation could act successfully as a biomarker of muscle function. (This idea would be consistent with the muscular weakness experienced by patients with muscular dystrophy, who also exhibit DGC deregulation). However, DGC deregulation was not associated significantly with weight loss. It is unclear if this lack of relationship to nutritional status was due simply to the relatively small number of muscle samples within the present thesis, as patients with DGC deregulation did have a higher median weight loss than patients with normal DGC (6.6% vs 0.2%, NS). Therefore, the question is still open as to whether or not DGC deregulation is an upstream mechanism of intracellular protein degradation within human skeletal muscle, or an end-organ effect (i.e. disruption of membrane stability) in its own right. At present, murine studies have suggested that

DGC deregulation might interact with the UPP (in an NF-κB-independent fashion) to induce muscle wasting [321], but similar mechanistic studies are required in humans to confirm this hypothesis.

The exact regulatory mechanisms underlying the reduced dystrophin expression and the hyperglycosylation of other DGC members are also unknown. Interestingly, in Western blots of patients with deregulated DGC, dystrophin expression appears to be effectively absent. A similar absence can also be observed in patients with muscular dystrophy [738]. Yet, in the face of weakened but intact muscle function, a complete absence of dystrophin might appear unlikely. The murine mAb reacts strongly with the carboxy terminus (between amino acids 3669 and 3685) of human dystrophin and shows no cross reactivity with *mdx* mouse tissue. Thus, it is unclear if dytrophin has been truly lost or if the epitope recognised by the antibody has been simply cleaved off.

Aberrant glycosylation of DGC members has been observed previously in specific forms of muscular dystrophy (e.g. muscle eye brain disease and Fukuyama congenital muscular dystrophy) [739]. However, these are predominantly hypoglycosylation events of α-DG, as a result of mutations in certain glycosyltransferase genes (protein O-mannosyl-transferase [POMT] 1; POMT2; protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase [POMGnT] 1; fukutin; fukutin-related protein [FKRP]; and like-glycosyltransferase [LARGE]) [740], resulting in defective interaction with the ligand laminin [739]. It is easy to understand how genetic mutations might cause protein hypoglycosylation, but it is

less simple to explain an acquired cause of hyperglycosylation. One hypothesis is that glycosyltransferase activity is increased as a response to circulating pro-cachectic mediators. Thus, such enzymes may represent druggable targets in cancer cachexia once identified. Interestingly, if the existence of a human homologue of PIF is proven conclusively, one might theorise that cancer cachexia is a syndrome of inappropriate hyperglycosylation, as a functional PIF would also require assembly of an extensive carbohydrate shell.

On univariate survival analysis, DGC deregulation was associated with shortened patient survival. In fact, only surgical patients who exhibited deregulated muscle DGC died during the follow up period of assessment. However, larger patient studies are required in order to eradicate any potential Type I errors and to allow multivariate modelling to confirm if DGC deregulation is an independent predictor of survival. A further question that can be answered by larger studies is the exact 'power' of the prognostic ability of DGC deregulation. In the current thesis, muscle samples were extracted from patients undergoing surgical resection with curative intent, and yet peri-operative DGC analysis appeared capable of predicting death in the face of potentially curable disease. CRP is one of the few other analytes that has demonstrated equivalent prognostic power in being able to predict post-operative survival in patients with OGC [88] and colorectal cancer [741]. At first glance, the concept of a marker that can predict death in the face of curable disease (on pre-operative staging investigations) appears contradictory. However, it is known that upper GI cancer has a high propensity for early recurrence even when histopathological examination of the resected specimen suggests an R0 resection

[742]. Pre/peri-operative elevated CRP and deregulated muscle DGC both imply a tumour that, although localised anatomically, is having systemic effects. Thus, it would not be surprising if these were the same patients who had sub-clinical micrometastases. Larger clinical studies linking DGC status to pathological/nodal status would be enlightening.

In summary, DGC deregulation can be found in patients with OGC, and it is likely that in larger clinical studies it will be confirmed as a marker of both cachexia and poor prognosis. Future studies might use ROC curves to assess the relationship between DGC deregulation and varying clinical definitions of cachexia. As DGC deregulation has already been proven conclusively (in the context of muscular dystrophy) to result in muscle weakness and increased fatigue, DGC deregulation might also represent a useful marker of patient functional status. This hypothesis is supported by the present thesis that demonstrated a trend towards worsened KPS in patients with deregulated DGC. If DGC deregulation were confirmed as a key mechanism in cachexia and an independent predictor of survival, some treatments being trialed for use in patients with muscular dystrophy would also be applicable to the treatment of cancer cachexia. Examples of medications and strategies include losartan (angiotensin II receptor anatagonist) [743], Protandim (anti-oxidant nutritional supplement) [744], techniques of utrophin upregulation [745], and pericyte stem cell replacement [746]. Doubt surrounds as to whether or not dystrophin gene replacement therapy, arguably the most discussed potential treatment for muscular dystrophy, would be beneficial (or ethically advisable) as, at present, it is unknown if gene therapy-induced supra-physiological levels of

dystrophin might be sufficient to overcome cachexia, or even if patients with cancer cachexia also have underlying genetic defects of dystrophin. To date, polymorphism studies of patients with cancer cachexia have not demonstrated any sequence variants of significance within the dystrophin gene, although genome-wide association studies are awaited.

Muscle mass is determined by the balance between protein synthesis and protein degradation. Intracellular signalling mechanisms of either process may be key targets for therapeutic intervention (see Figure 1.11, p.101, and Figure 1.12, p.102). PKR is a serine/threonine specific protein kinase, which undergoes autophosphorylation and activation, in the presence of interferon, in response to viral attack [636] (see Figure 8.1). Activated PKR phosphorylates several protein substrates including the α-subunit of eIF2 [637], resulting in inhibition of protein synthesis in pre-clinical models [190]. Furthermore, phosphorylated PKR has been shown to induce NF-κB-dependent protein degradation via the UPP *in vitro* [188, 190, 652]. In Chapter 8, increased phospho forms of PKR and eIF2α were detected in rectus abdominis samples from weight-losing patients with OGC (n=15) compared with healthy controls. Phospho PKR levels correlated with phospho eIF2α levels, suggesting that PKR phosphorylation leads directly to eIF2α phosphorylation. Moreover, phospho eIF2α levels correlated with myosin levels, suggesting that PKR and eIF2α are intermediates in pathways leading to increased protein degradation within skeletal muscle. Further studies to confirm mechanistic links between PKR, NF-κB and the UPP have not yet been performed in human muscle.

Although the present study confirmed a clear alteration in phosphorylated mediator levels between weight-losing cancer patients and controls, there did not appear to be a correlation between the degree of weight loss and the level of mediator phosphorylation. In studies using murine MAC16 cachexia model, these changes appeared to be progressive [652]. Furthermore, studies of other intramuscular mediators of protein degradation have shown that as weight loss increases, mediator levels reach a maximum peak before falling again [360]. One reason for this phenomenon is that depression of protein synthesis may become more influential than induction of protein degradation at severe/refractory stages of cachexia [360]. These observational differences may simply reflect differences between murine models and the human condition, but further studies incorporating larger numbers of patients with extreme weight loss would help to confirm this.

The present thesis represents an association study and, as of yet, there are no data regarding the upstream or downstream mediators of PKR phosphorylation. In murine and *in vitro* models, PIF and angiotensin II have been proposed as the upstream inducers [188, 190]. However, at present, there is no strong evidence for a role for either of these mediators in human cancer cachexia. It would make sense to combine future studies of PKR phosphorylation with DGC deregulation as both were investigated in the same tissue type (namely, rectus abdominis skeletal muscle samples from OGC patients) within the present thesis. In this way, it would be interesting to investigate if phosphorylation of PKR/eIF2α is an intermediate downstream step of deregulated DGC-induced protein degradation. Comparative

studies of trunk with limb muscle groups would offer information on differences between weight-bearing and non-weight-bearing muscles.

In summary, phospho PKR and phospho eIF2α would both appear to have potential as future biomarkers of skeletal muscle wasting, as elevated levels were even observed in some patients who had not yet experienced weight loss. Phosphorylated mediator levels might also represent an effective marker of patient response to anti-cachexia therapeutic intervention. However, such levels might only be used in a "yes/no" fashion, and may not predict accurately or correlate with the severity of cachexia experienced. Inhibitors of PKR, such as the Ca^{2+} chelator 1,2-bis (o-aminphenoxy) ethane-N,N,N',N'-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA/AM) [190], D-myo-inositol 1,2,6-triphosphate [747], HMB [413], and potentially, insulin and IGF-1[748], may be prospective therapeutic agents for cancer cachexia.

In Chapters 7 and 8, intramuscular metabolic changes induced by cancer cachexia were analysed. However, in Chapter 9, the subject of focus was altered to the investigation of the end-organ effects of skeletal muscle wasting. Specifically, MS analysis of human urine was performed in order to identify candidate protein biomarkers of cachexia, including breakdown products of skeletal muscle protein degradation. (The proposed hypothesis underlying the existence of cachectic biomarkers in human urine is shown in Figure 12.4). In particular, species of myosin (including myosins 5C, 7A, heavy polypeptide 7, 9A, and 10) were identified specifically in urine samples from OGC patients with cancer cachexia

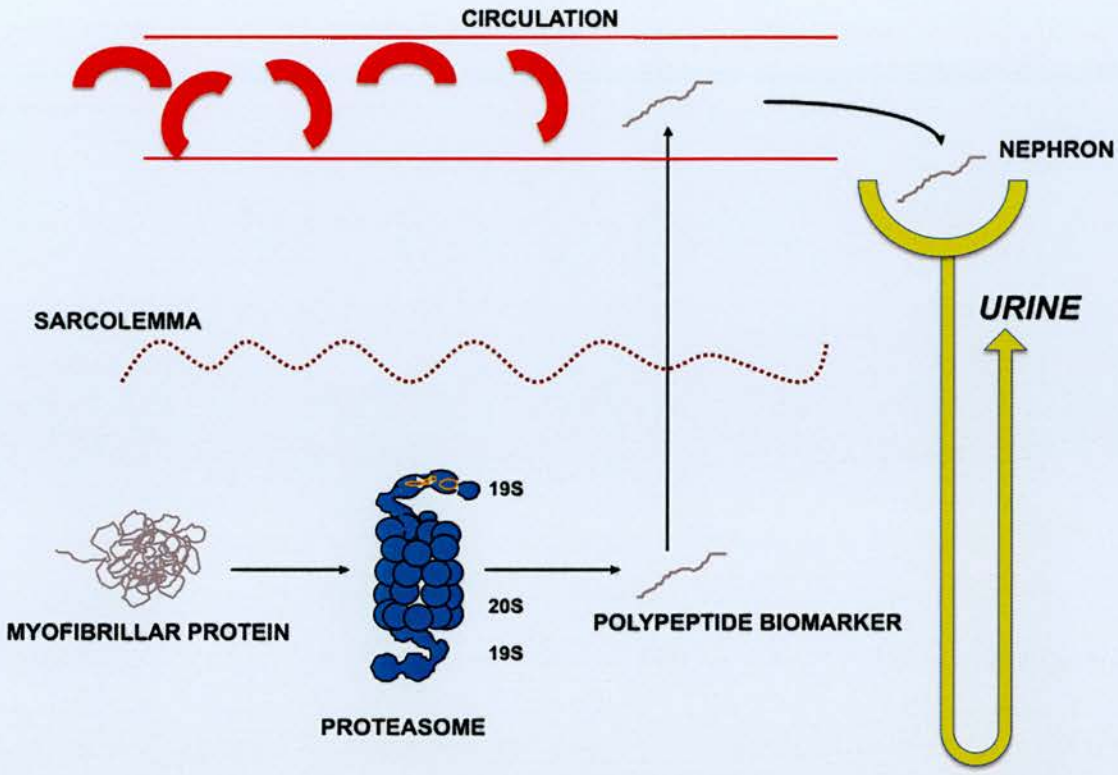

Figure 12.4 **Proposed mechanism for the existence of biomarkers of skeletal muscle wasting in cachectic human urine.**
Myofibrillar proteins such as myosin are degraded by components of the ubiquitin proteasome pathway. These smaller polypeptide fragments (representing biomarkers) either leach out or are actively excreted by muscle cells through the increasingly permeable sarcolemma. (Permeability is increased during cancer cachexia through mechanisms including DGC deregulation). The polypeptides enter the circulation and are filtered by the nephron to be excreted in urine from whence they are detected. The exact size of the polypeptide biomarkers and the mechanisms of ultrafiltration by the kidney require further elucidation.

(n=8; weight loss ≥10%) compared with weight-stable cancer patients (n=8) and healthy controls (n=8), thus confirming the presence of myofibrillar degradation. Despite the predominance of skeletal muscle wasting in cancer cachexia, the present thesis represents the first identification of muscle breakdown products in patient urine. Previous studies have focused simply on the detection of non-specific proteinuria [659] or nitrogen excretion [658].

In the present thesis, the one conventional myosin detected specifically in cachectic urine was MyHC 7/cardiac muscle/β variant, a component of type I skeletal muscle fibres and cardiac muscle [749]. However, in Chapter 7, it was described how myofibrillar ATPase staining demonstrated selective atrophy of fast twitch type II muscle fibres in the C-26 mouse model [321]. Furthermore, similar fibre type-specific wasting is thought to occur during human cancer cachexia. Thus, the proposal of type I fibre wasting might appear at odds with some previous studies. However, other investigations have also demonstrated slight reductions in type I MyHC within cachectic muscle [321] and switching of fibre type from I to IIa in animal tumour models [691]. Chapter 7 also described how some changes of DGC deregulation were not limited to type II fibres, and that a faint hyperglycosylated form of β-DG and limited reduction of dystrophin expression were detectable in slow, oxidative, type I fibres also [321]. Thus, although atrophy might occur predominantly in type II fibres, some atrophy must also occur in type I fibres. Taken together though, the data from this thesis demonstrate reduced intramuscular myosin expression (Chapter 8) and actin-myosin complex association (Chapter 7) within cachectic human muscle, and the presence of myosin within cachectic

human urine (Chapter 9). These findings appear to confirm the presence of skeletal muscle wasting, and also support the choice of myosin as a protein worthy of investigation as a possible biomarker of cachexia. From the patient's perspective, urinary analysis of myosin by MS would be preferential to muscle biopsy (in the absence of resectional surgery).

Other potential biomarkers of cachexia detected in patient urine included bullous pemphigoid antigen 1, chromosome 14 open reading frame 78, MIBP1, zinc finger protein 106 homologue, nischarin, MACF1, MAP1B, and α1-spectrin. Several of these markers, particularly the latter three point to a disruption of basic cell structure as a key process within cancer cachexia. Future observational studies of human muscle samples should target these other potential biomarkers in order to confirm intramuscular depletion in cachectic patients.

In Chapter 7, it was described how muscles from the C-26 mouse demonstrated free uptake of EBD even though serum CK levels were only elevated moderately over controls [321]. Furthermore, in the same chapter, sarcolemmal membrane abnormalities (in the form of DGC deregulation) were confirmed in muscle samples from patients with OGC. The detection of myosin and other structural proteins in the urine of patients with the same disease, in the absence of plasma CK rise, implies that membrane damage during cachexia is not sufficient to cause a complete permeation of intracellular proteins. Rather these data point to a selective loss of ultrastructure in the presence of mild membrane damage. Data from *in vitro*

systems and the C-26 model, which show selective targeting of MyHC during cancer cachexia [46], support this hypothesis.

In the present thesis, MS analysis was performed following tryptic digestion of urinary protein. Database searching was then performed for whole proteins on a probability basis. Thus, biomarker identification is dependent on statistical probability, and therefore some of the listed cachectic biomarkers may be inaccurate or redundant. Future proteomic studies that are capable of identifying accurately the peptide fragments released by cachectic muscle will not only refine the number and nature of the biomarkers, but also the size of the fragments. In this way, conclusions may be drawn regarding whether such fragments simply leach out of cachectic fibres or whether active transport mechanisms are involved, and how the kidneys filter such fragments.

It is important to note that myoglobin was not detected in the urine of cachectic cancer patients. If cancer cachexia in patients with OGC mirrored muscular dystrophy exactly, one might not only expect a rise in plasma CK in some patients, but also a rise in plasma and urinary myoglobin. However, previous studies have shown that plasma levels of myoglobin are reduced by approximately 30% in cachectic cancer patients compared with controls, and that plasma myoglobin correlates with quadriceps cross-sectional area and the area fraction formed by type I and IIa fibres [657]. Clearly, differences do exist in the mechanistic pathways of muscle protein degradation and elimination between cancer cachexia and muscular dystrophy.

A human homologue of PIF was not identified in the cachectic patient urine using the MS techniques employed in the current thesis. (Urine has been the predominant patient sample used for the demonstration of apparent PIF immunoreactivity with the PIF mAb [183]). However, interestingly, ZAG, the putative human LMF [220], was detected in the urine of several of the cancer patients, both cachectic and weight-stable. One could argue that this finding suggests that LMF production and fat depletion occurs early in cachexia, whereas PIF production and muscle wasting occurs later.

The ultimate aim of biomarker research is to identify individual markers or patterns of markers that can be used either as inclusion criteria for clinical trials or can be used to monitor response to therapy. Furthermore, the degradative pathways involved in the early loss of myosin are a major area of current research in cancer cachexia. In this preliminary proof-of-principle study, urinary myosin has shown promise as a potential biomarker of skeletal muscle wasting. The next step should seek to use a quantitative MS methodology (rather than the qualititative approach used in the present thesis) in the context of a longitudinal study in order to measure the changing fractions of urinary biomarkers over time, or pre- and post-intervention. A different search strategy that employs identification of fragmented peptide sequences rather than probability-based extrapolation of the original progenitor molecule might also improve accuracy and reduce redundancy.

In cachexia intervention trials, the FDA has stated that measures of patient PF are required alongside measures of body composition (e.g. LBM/skeletal muscle mass)

[466]. In Chapters 10 and 11, the subject of the present thesis moved away from the analysis of molecular mediators and markers of muscle wasting, and focused on muscle function and its potential as a clinical biomarker for cachexia studies. Global muscle (and therefore patient) function was assessed using the activPAL™ meter, a lightweight accelerometer-based device worn on the patient thigh that offers information on long-term, everyday PF within the home/outpatient environment. ActivPAL™ offers a wide range of simultaneous outcome measures (e.g. TEE, EEA, time spent sitting/lying, time spent standing, step count, step cadence), each of which can be analysed during a single recording episode. Furthermore, some of the simpler outcomes have been validated already in healthy controls [708, 709]. The activPAL™ meter is user/patient friendly and economically viable, thus offering significant advantages over the gold standard methodology for assessing PA (i.e. DLW). The major outputs from the activPAL™ meter are estimates of TEE and EEA. These outcome measures are of importance in cancer cachexia for several reasons. Firstly, although REE may be increased in some wasted cancer patients, TEE may actually fall suggesting that they reduce energy demand by decreasing PA, and hence EEA [277]. Secondly, and importantly, it has been shown that objective measures of PA (PAL and EEA) can be improved by anti-cachexia interventions (i.e. EPA) [277]. Lastly, as hypothesised in the present thesis, there is emerging evidence that objective measures of PA correlate with health-related QoL scores [380, 513]. Thus, intervention-induced improvements in patient PA should translate into improvements in QoL, which in the context of advanced cancer patients with short life expectancy is a significant benefit. Figure 12.5 demonstrates the varied

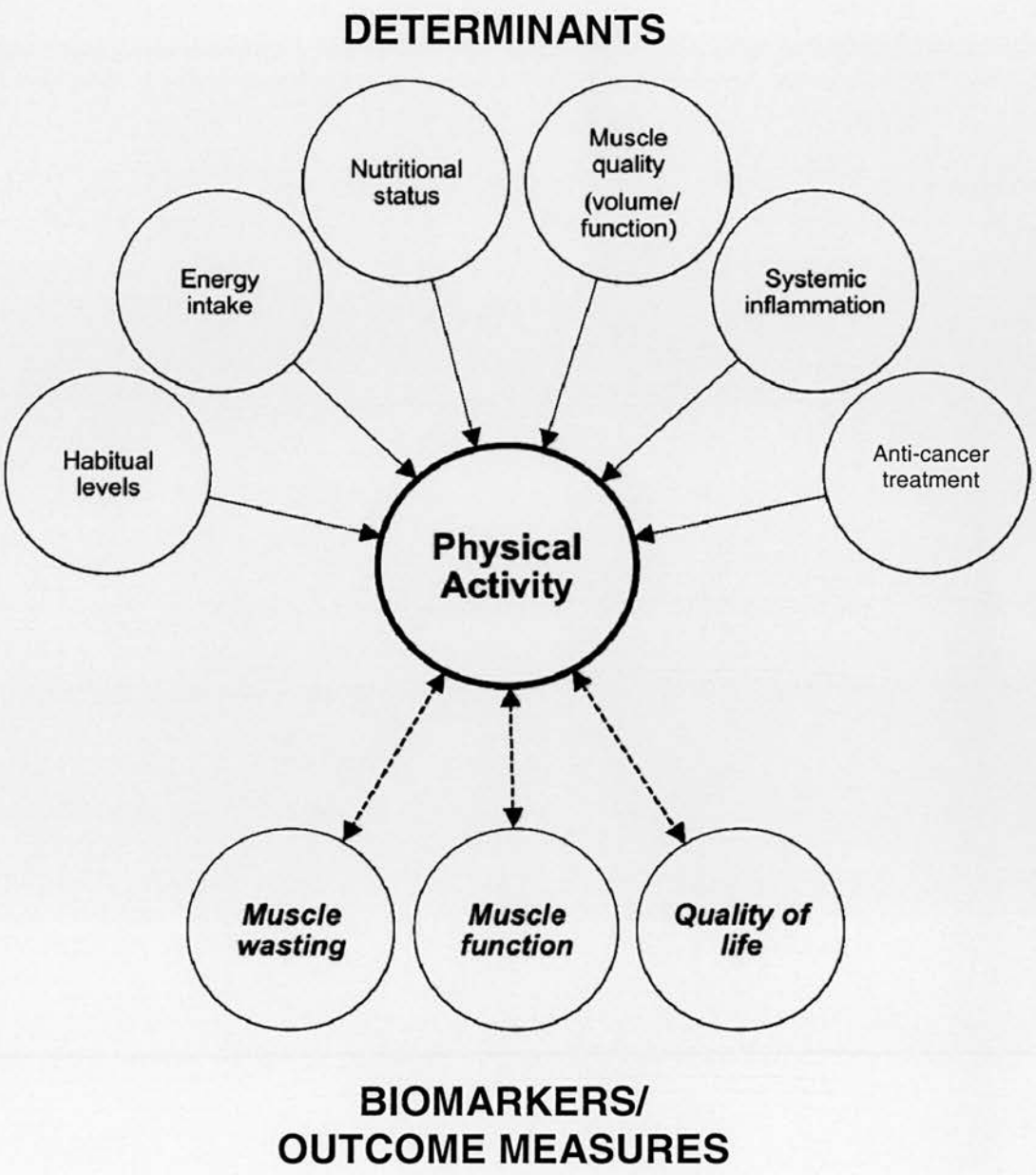

Figure 12.5 **The determinants of objective physical activity, and the relationship with muscle function, muscle wasting and quality of life.** A patient's physical activity is influenced by a number of patient- and disease-related factors. However, the objective assessment of physical activity will offer information on that individual's muscle function, muscle wasting and quality of life.

determinants of a patient's PA, and the relationship PA shares with muscle function, muscle wasting and QoL.

In Chapter 10, a 2-week DLW protocol, in combination with indirect calorimetry, was used to validate the EE output of the activPAL™ meter in a cohort of advanced cancer patients (n=6) and healthy controls (n=9 assessments in 8 subjects). As expected, when assessed by DLW, cancer patients exhibited lower mean TEE and EEA compared with healthy subjects. Across the whole experimental group, average measures of TEE and EEA were validated with only very small mean absolute errors (0.4% and.1.4%, respectively), although LOA were wide enough to question the applicability of activPAL™-derived measures of EE in individual patients (see Figure 10.4, p.351, and Figure 10.5, p.353). Further larger studies are required to validate accurately the EE outputs of activPAL™.

TEE and EEA were chosen initially as the PA outcomes of choice, as they represent global assessments of muscle function, incorporating all muscle activity. TEE also incorporates REE, which may be increased during cancer cachexia [550]. However, EE outputs may not be the most sensitive measures of patient function. Simpler measures of PF such as step count correlated well with EEA, and thus alternative outcomes that are easier to assess by simpler devices (e.g. pedometers) may exist, and may be superior in terms of sensitivity. However, the question remains why, if EEA appears to be related intimately to step count, did activPAL™-derived measures of EEA demonstrate high variability? Recent Norwegian data has suggested that the validity of activPAL™-derived step count may vary according to

walking speed (personal communication: Guro Stene), and that frailer, more cachectic cancer patients demonstrate greater step count inaccuracy [712]. Assuming that PA meter validity may vary according to walking speed implies that validation studies of outcome measures should only be carried out in the specific patient population to which they will be applied, and that no studies of mixed populations should occur. Thus, any validation data extracted from a certain patient population cannot be transferred for use in another. This might be levelled as a criticism against the present thesis, which used a mixed population of cancer patients and healthy controls. However, despite the inherent difficulties, validation studies remain of paramount importance in the development of accurate outcome measures for trial use. As has been shown in the present thesis, assumptions that underpin any device may be incorrect biologically when applied to a specific population (e.g. the calorific value of 1 activPAL™-derived MET actually appeared to be 0.84kcal/kg/hr within the present study cohort).

In summary, the activPAL™ meter can offer objective data on TEE and EEA in patients with advanced cancer and healthy controls, although the variable validity of this data precludes immediate use in clinical trials as a biomarker of muscle function, pending further studies. Validation studies of other simpler activPAL™-derived measures may offer more accurate, but possibly less informative outcomes in the interim period.

In any future therapeutic strategy for cancer cachexia, it is likely that many of the patients will also be receiving simultaneous palliative chemotherapy. Although

such chemotherapy is often administered with the long-term aim of improving patient PS, during the period of treatment, it is often associated with unwanted side effects and a negative impact on QoL. Furthermore, some chemotherapeutic agents are known to exacerbate skeletal muscle wasting [290]. Therefore, any future outcome measure for use in cachexia intervention trials must be applicable within the context of palliative chemotherapy. In Chapter 11, activPAL™ was used to perform sequential assessments of PA in a cohort of upper GI cancer patients receiving palliative chemotherapy (n=16). Furthermore, QoL questionnaires were completed to assess the relationship between PA and QoL. The present thesis demonstrated an overall trend towards reduction in PA throughout chemotherapy (although individual patients described a complex PA journey with some demonstrating reduced, increased or unchanged PA). In general, results did not achieve statistical significance, although time spent stepping did increase significantly as the duration of time to cessation of treatment increased. It remains an important finding in its own right, however, that activPAL™ was capable of detecting demonstrable changes in the objective PA of individual patients in response to treatment and over time.

Objective measures of PA, including time spent sitting or lying, time spent standing, time spent upright and EEA correlated not only with subjective measures of PS and questionnaire-derived measures of PWB and FWB, but also with EORTC QLQ-C30 Global Health Status/QoL and mood (HADS-Depression score). This is a contrast to previous studies that have not been able to confirm a relationship between PA and global QoL. The present thesis helps to justify the use of objective

PA as an outcome measure in future clinical trials of anti-cachexia therapies, and supports the concept that intervention-induced improvement in PA will cause a coincident improvement in QoL.

Although objective PA measures correlated significantly with subjective PS scores, they highlighted the inherent inaccuracy and variability of PS. For example, patients with WHO PS score 1 exhibited step counts of between 1007 and 7619 steps/day i.e. patients at the upper end of the WHO PS 1 category performed over 7 times the objective PA of patients at the lower end. Thus, it seems illogical that such varying patients should be considered to have equivalent levels of PF. Clearly, objective assessment of PA does hold some advantages over subjective scoring of PS.

The present data also calls into question the ability of health-related QoL to model accurately symptoms of cancer cachexia. PA measures correlated inversely with percentage weight loss, an objective marker of altered body composition, and yet they did not correlate with the FAACT ACS score. Furthermore, although patients actually demonstrated significant reductions in weight, AMC and body fat following initiation of chemotherapy, the ACS score was shown to improve in a contradictory fashion. Thus, current self-reported scores of patient symptomatology remain inferior to true measures of body composition during the diagnosis and classification of cachexia.

Importantly, time spent upright correlated with LBM as a percentage of total body weight, thus demonstrating that PA and muscle mass are linked, and that reduced PA should be a good surrogate marker of skeletal muscle wasting as well as function. Morover, any intervention that increases muscle mass should improve PA, and potentially, vice-versa. Ongoing studies comparing measures of muscle strength/power and PA with MRI analysis of muscle volume are being perfomed to investigate further the relationship between function and size.

One difficulty that has become apparent when analysing this sort of study is how to factor in clinical events that occur within heterogenous populations of cancer patients and that are bound to impact on patient PA (e.g. different types of cancer; different types of chemotherapy regimen; chemotherapy side effects; blood transfusion; oesophageal stenting). Preliminary trials using objective PA as an outcome measure may need to specify patient homogeneity as much as possible within inclusion criteria in order to mitigate any unwanted and confusing variability in PA. Strict recording of both clinical events and tumour response to chemotherapy will be required to tease apart the separate effects of disease, chemotherapy and anti-cachexia intervention on patient PA.

In summary, objective measures of PA (derived using the activPAL™ meter) offer data on global patient/muscle function during palliative chemotherapy, and correlate significantly with both QoL and body composition. They also underline the subjectivity and inaccuracies of both PS scores and some self-reported symptom

scores. As markers of muscle function, PA measures should be considered as outcomes in future trials of anti-cachexia interventions.

In the above section, the findings of the present thesis have been brought together, and suggestions for future investigations have been made in order to answer some of the remaining questions in cancer cachexia. However, in the next section, actual funded research studies that have already been initiated are described briefly.

12.2 Ongoing research

The identification of markers for early intervention, data linking markers and mediators with specific changes in skeletal muscle volume and function, and the development of an effective multimodal treatment still represent clear unmet clinical needs in cancer cachexia. Thus, two research programmes (funded by CRUK and Wyeth, respectively) have been commenced, the specific aims of which are to identify early markers of cachexia and relate these to longitudinal measures of skeletal muscle volume and function. Building on the concept of 'pre-cachexia', these studies will incorporate some of the experimental suggestions detailed above, and are are based on the following hypotheses:

1. Cachexia is a progressive condition that begins prior to gross muscle wasting and weight loss, and that this early phase can be identified from phenotypic changes in blood, urine and skeletal muscle.

2. There are early changes in skeletal muscle function (ahead of wasting), and these changes can be related to biochemical markers of cachexia.

Upper GI cancer patients represent the investigative model of choice, and tissues being studied include skeletal muscle, blood, urine, tumour and fat. The mediators, mechanisms and biomarkers of skeletal muscle wasting and function that are being employed in these newer studies are those with proven importance in the present thesis:

- Sex steroids and gonadotropins
- DGC deregulation
- PKR/eIF2α phosphorylation and other mechanisms of reduced protein synthesis and increased degradation
- Urinary biomarkers of muscle wasting, including myosin
- ActivPAL™-derived measures of PA, as markers of muscle function

12.3 Final conclusions

The present thesis has explored a number of different themes surrounding the syndrome of cancer cachexia. The final conclusions of the thesis regarding mediators, mechanisms and biomarkers of skeletal muscle wasting and function in human cancer cachexia are:

- A putative human homologue of PIF is yet to be proven as an important factor in human cancer cachexia.
- MIC-1 does not appear to regulate significantly nutritional depletion or skeletal muscle wasting in cancer cachexia.
- Sexually dimorphic alterations in patient gonadal status may influence nutritional status and outcome in cancer cachexia.
- DGC deregulation and PKR/eIF2α activation play a role in skeletal muscle wasting in cancer cachexia and represent novel therapeutic targets.
- Urinary biomarkers (e.g. myosin) of skeletal muscle wasting can be detected in patients with cancer and should be developed further as potential biomarkers of the cachexia syndrome.
- Objective PA as a marker of skeletal muscle function is a complex endpoint that is influenced by numerous factors (including chemotherapy) but which has inherent value within trial methodology.

Combined with findings from the published literature, extrapolation of these conclusions to the hypothetical development of an ideal multimodal intervention trial in cancer cachexia would suggest that any such trial might include the following components:

- Experimental multimodal therapy
  - nutritional support
  - anti-inflammatories
  - *neuroendocrine modulation (e.g. androgen replacement therapy*

in men, oestradiol suppression in women)

- *anabolic muscle agents (e.g. dystrophin replacement, PKR inhibitors)*
- appetite stimulants
- physical exercise

- Inclusion criteria
  - weight loss/reduced skeletal muscle mass
  - systemic inflammation
  - *presence or raised levels of urinary biomarkers (e.g. myosin)*

- Outcome measures
  - nutritional status
  - *objective assessment of PA (as marker of skeletal muscle function and QoL)*
  - *levels of urinary biomarkers (e.g. myosin)*
  - survival

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Appendix I – Buffers

TAE X40

- Tris 193.6g
- Na acetate.$3H_2O$ 108.9g
- diNaEDTA.$2H_2O$ 15.2g

pH to 7.2 with acetic acid
Water to 1 litre

TBE 5X

- Tris 53.9g
- EDTA 3.72g
- Boric acid 30g boric acid

1.6% agarose gel for PCR

- LE agarose 1.6g
- 1XTAE 100ml

Run in 1XTAE at 80 volts

6% acrylamide gel for PCR

- 40% acrylamide soln (29:1) (Sigma) 3ml
- dH_2O 12.8ml
- 5XTBE 4ml
- 10% AMPS 140µl
- TEMED 30µl

Run in 1XTBE at 200 volts

Low molecular weight markers
Reconstitute in 200µl loading buffer for markers (in fume hood)
Heat @ 95°C for 5mins
Aliquot-5µl

High molecular weight markers
Reconstitute in 100µl H_2O
Do not heat
Aliquot-5µl

Appendix II – Performance scores

Karnofsky performance score

- 100% - normal, no complaints, no signs of disease
- 90% - capable of normal activity, few symptoms or signs of disease
- 80% - normal activity with some difficulty, some symptoms or signs
- 70% - caring for self, not capable of normal activity or work
- 60% - requiring some help, can take care of most personal requirements
- 50% - requires help often, requires frequent medical care
- 40% - disabled, requires special care and help
- 30% - severely disabled, hospital admission indicated but no risk of death
- 20% - very ill, urgently requiring admission, requires supportive measures or treatment
- 10% - moribund, rapidly progressive fatal disease processes
- 0% - death

World Health Organisation performance score

- 0 - asymptomatic (fully active, able to carry on all pre-illness activities without restriction)
- 1 - symptomatic but completely ambulatory (restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature e.g. light housework, office work)
- 2 - symptomatic, <50% in bed during the day (ambulatory and capable of all self care but unable to carry out any work activities. Up and about more than 50% of waking hours)
- 3 - symptomatic, >50% in bed, but not bedbound (capable of only limited self-care, confined to bed or chair 50% or more of waking hours)
- 4 - bedbound (completely disabled, cannot carry on any self-care, totally confined to bed or chair)
- 5 - death

Appendix III – Questionnaires

1. European Organisation for Research and Treatment of Cancer QLQ-C30 (EORTC QLQ-C30)
2. Functional Assessment of Anorexia/Cachexia Therapy (FAACT)
3. Functional Assessment of Chronic Illness Therapy - Fatigue (FACIT-F)
4. Hospital Anxiety and Depression Scale (HADS)

EORTC QLQ-C30 (version 3)

We are interested in some things about you and your health. Please answer all of the questions yourself by circling the number that best applies to you. There are no "right" or "wrong" answers. The information that you provide will remain strictly confidential.

Please fill in your initials: ⌊_⌊_⌊_⌊_⌋

Your birthdate (Day, Month, Year): ⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌋

Today's date (Day, Month, Year): 31 ⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌋

	Not at All	A Little	Quite a Bit	Very Much
1. Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	1	2	3	4
2. Do you have any trouble taking a long walk?	1	2	3	4
3. Do you have any trouble taking a short walk outside of the house?	1	2	3	4
4. Do you need to stay in bed or a chair during the day?	1	2	3	4
5. Do you need help with eating, dressing, washing yourself or using the toilet?	1	2	3	4

During the past week:

	Not at All	A Little	Quite a Bit	Very Much
6. Were you limited in doing either your work or other daily activities?	1	2	3	4
7. Were you limited in pursuing your hobbies or other leisure time activities?	1	2	3	4
8. Were you short of breath?	1	2	3	4
9. Have you had pain?	1	2	3	4
10. Did you need to rest?	1	2	3	4
11. Have you had trouble sleeping?	1	2	3	4
12. Have you felt weak?	1	2	3	4
13. Have you lacked appetite?	1	2	3	4
14. Have you felt nauseated?	1	2	3	4
15. Have you vomited?	1	2	3	4
16. Have you been constipated?	1	2	3	4

Please go on to the next page

During the past week:	Not at All	A Little	Quite a Bit	Very Much
17. Have you had diarrhea?	1	2	3	4
18. Were you tired?	1	2	3	4
19. Did pain interfere with your daily activities?	1	2	3	4
20. Have you had difficulty in concentrating on things, like reading a newspaper or watching television?	1	2	3	4
21. Did you feel tense?	1	2	3	4
22. Did you worry?	1	2	3	4
23. Did you feel irritable?	1	2	3	4
24. Did you feel depressed?	1	2	3	4
25. Have you had difficulty remembering things?	1	2	3	4
26. Has your physical condition or medical treatment interfered with your family life?	1	2	3	4
27. Has your physical condition or medical treatment interfered with your social activities?	1	2	3	4
28. Has your physical condition or medical treatment caused you financial difficulties?	1	2	3	4

For the following questions please circle the number between 1 and 7 that best applies to you

29. How would you rate your overall health during the past week?

1	2	3	4	5	6	7
Very poor						Excellent

30. How would you rate your overall quality of life during the past week?

1	2	3	4	5	6	7
Very poor						Excellent

FAACT (Version 4)

Below is a list of statements that other people with your illness have said are important. **Please circle or mark one number per line to indicate your response as it applies to the <u>past 7 days</u>.**

	PHYSICAL WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GP1	I have a lack of energy	0	1	2	3	4
GP2	I have nausea	0	1	2	3	4
GP3	Because of my physical condition, I have trouble meeting the needs of my family	0	1	2	3	4
GP4	I have pain	0	1	2	3	4
GP5	I am bothered by side effects of treatment	0	1	2	3	4
GP6	I feel ill	0	1	2	3	4
GP7	I am forced to spend time in bed	0	1	2	3	4

	SOCIAL/FAMILY WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GS1	I feel close to my friends	0	1	2	3	4
GS2	I get emotional support from my family	0	1	2	3	4
GS3	I get support from my friends	0	1	2	3	4
GS4	My family has accepted my illness	0	1	2	3	4
GS5	I am satisfied with family communication about my illness	0	1	2	3	4
GS6	I feel close to my partner (or the person who is my main support)	0	1	2	3	4
Q1	*Regardless of your current level of sexual activity, please answer the following question. If you prefer not to answer it, please mark this box* ☐ *and go to the next section.*					
GS7	I am satisfied with my sex life	0	1	2	3	4

FAACT (Version 4)

Please circle or mark one number per line to indicate your response as it applies to the <u>past 7 days</u>.

	EMOTIONAL WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GE1	I feel sad	0	1	2	3	4
GE2	I am satisfied with how I am coping with my illness	0	1	2	3	4
GE3	I am losing hope in the fight against my illness	0	1	2	3	4
GE4	I feel nervous	0	1	2	3	4
GE5	I worry about dying	0	1	2	3	4
GE6	I worry that my condition will get worse	0	1	2	3	4

	FUNCTIONAL WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GF1	I am able to work (include work at home)	0	1	2	3	4
GF2	My work (include work at home) is fulfilling	0	1	2	3	4
GF3	I am able to enjoy life	0	1	2	3	4
GF4	I have accepted my illness	0	1	2	3	4
GF5	I am sleeping well	0	1	2	3	4
GF6	I am enjoying the things I usually do for fun	0	1	2	3	4
GF7	I am content with the quality of my life right now	0	1	2	3	4

FAACT (Version 4)

Please circle or mark one number per line to indicate your response as it applies to the <u>past 7 days</u>.

	ADDITIONAL CONCERNS	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
C6	I have a good appetite	0	1	2	3	4
ACT1	The amount I eat is sufficient to meet my needs	0	1	2	3	4
ACT2	I am worried about my weight	0	1	2	3	4
ACT3	Most food tastes unpleasant to me	0	1	2	3	4
ACT4	I am concerned about how thin I look	0	1	2	3	4
ACT6	My interest in food drops as soon as I try to eat	0	1	2	3	4
ACT7	I have difficulty eating rich or "heavy" foods	0	1	2	3	4
ACT9	My family or friends are pressuring me to eat	0	1	2	3	4
O2	I have been vomiting	0	1	2	3	4
ACT10	When I eat, I seem to get full quickly	0	1	2	3	4
ACT11	I have pain in my stomach area	0	1	2	3	4
ACT13	My general health is improving	0	1	2	3	4

FACIT-F (Version 4)

Below is a list of statements that other people with your illness have said are important. **Please circle or mark one number per line to indicate your response as it applies to the <u>past 7 days</u>.**

	PHYSICAL WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GP1	I have a lack of energy	0	1	2	3	4
GP2	I have nausea	0	1	2	3	4
GP3	Because of my physical condition, I have trouble meeting the needs of my family	0	1	2	3	4
GP4	I have pain	0	1	2	3	4
GP5	I am bothered by side effects of treatment	0	1	2	3	4
GP6	I feel ill	0	1	2	3	4
GP7	I am forced to spend time in bed	0	1	2	3	4

	SOCIAL/FAMILY WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GS1	I feel close to my friends	0	1	2	3	4
GS2	I get emotional support from my family	0	1	2	3	4
GS3	I get support from my friends	0	1	2	3	4
GS4	My family has accepted my illness	0	1	2	3	4
GS5	I am satisfied with family communication about my illness	0	1	2	3	4
GS6	I feel close to my partner (or the person who is my main support)	0	1	2	3	4
Q1	*Regardless of your current level of sexual activity, please answer the following question. If you prefer not to answer it, please mark this box* ☐ *and go to the next section.*					
GS7	I am satisfied with my sex life	0	1	2	3	4

FACIT-F (Version 4)

Please circle or mark one number per line to indicate your response as it applies to the <u>past 7 days</u>.

	EMOTIONAL WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GE1	I feel sad	0	1	2	3	4
GE2	I am satisfied with how I am coping with my illness	0	1	2	3	4
GE3	I am losing hope in the fight against my illness	0	1	2	3	4
GE4	I feel nervous	0	1	2	3	4
GE5	I worry about dying	0	1	2	3	4
GE6	I worry that my condition will get worse	0	1	2	3	4

	FUNCTIONAL WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GF1	I am able to work (include work at home)	0	1	2	3	4
GF2	My work (include work at home) is fulfilling	0	1	2	3	4
GF3	I am able to enjoy life	0	1	2	3	4
GF4	I have accepted my illness	0	1	2	3	4
GF5	I am sleeping well	0	1	2	3	4
GF6	I am enjoying the things I usually do for fun	0	1	2	3	4
GF7	I am content with the quality of my life right now	0	1	2	3	4

FACIT-F (Version 4)

Please circle or mark one number per line to indicate your response as it applies to the past 7 days.

	ADDITIONAL CONCERNS	Not at all	A little bit	Some-what	Quite a bit	Very much
HI7	I feel fatigued	0	1	2	3	4
HI12	I feel weak all over	0	1	2	3	4
An1	I feel listless ("washed out")	0	1	2	3	4
An2	I feel tired	0	1	2	3	4
An3	I have trouble starting things because I am tired	0	1	2	3	4
An4	I have trouble finishing things because I am tired	0	1	2	3	4
An5	I have energy	0	1	2	3	4
An7	I am able to do my usual activities	0	1	2	3	4
An8	I need to sleep during the day	0	1	2	3	4
An12	I am too tired to eat	0	1	2	3	4
An14	I need help doing my usual activities	0	1	2	3	4
An15	I am frustrated by being too tired to do the things I want to do	0	1	2	3	4
An16	I have to limit my social activity because I am tired	0	1	2	3	4

Hospital Anxiety and Depression Scale (HADS)

Patients are asked to choose one response from the four given for each interview. They should give an immediate response and be dissuaded from thinking too long about their answers. The questions relating to anxiety are marked "A", and to depression "D". The score for each answer is given in the right column. Instruct the patient to answer how it currently describes their feelings.

A	**I feel tense or 'wound up':**	
	Most of the time	3
	A lot of the time	2
	From time to time, occasionally	1
	Not at all	0

D	**I still enjoy the things I used to enjoy:**	
	Definitely as much	0
	Not quite so much	1
	Only a little	2
	Hardly at all	3

A	**I get a sort of frightened feeling as if something awful is about to happen:**	
	Very definitely and quite badly	3
	Yes, but not too badly	2
	A little, but it doesn't worry me	1
	Not at all	0

D	**I can laugh and see the funny side of things:**	
	As much as I always could	0
	Not quite so much now	1
	Definitely not so much now	2
	Not at all	3

A	**Worrying thoughts go through my mind:**	
	A great deal of the time	3
	A lot of the time	2
	From time to time, but not too often	1
	Only occasionally	0

D	**I feel cheerful:**	
	Not at all	3
	Not often	2
	Sometimes	1
	Most of the time	0

A	**I can sit at ease and feel relaxed:**	
	Definitely	0
	Usually	1
	Not Often	2
	Not at all	3

D	**I feel as if I am slowed down:**	
	Nearly all the time	3
	Very often	2
	Sometimes	1
	Not at all	0

A	**I get a sort of frightened feeling like 'butterflies' in the stomach:**	
	Not at all	0
	Occasionally	1
	Quite Often	2
	Very Often	3

D	**I have lost interest in my appearance:**	
	Definitely	3
	I don't take as much care as I should	2
	I may not take quite as much care	1
	I take just as much care as ever	0

A	**I feel restless as I have to be on the move:**	
	Very much indeed	3
	Quite a lot	2
	Not very much	1
	Not at all	0

D	**I look forward with enjoyment to things:**	
	As much as I ever did	0
	Rather less than I used to	1
	Definitely less than I used to	2
	Hardly at all	3

A	**I get sudden feelings of panic:**	
	Very often indeed	3
	Quite often	2
	Not very often	1
	Not at all	0

D	**I can enjoy a good book or radio or TV program:**	
	Often	0
	Sometimes	1
	Not often	2
	Very seldom	3

	Scoring (add the As = Anxiety. Add the Ds = Depression). The norms below will give you an idea of the level of Anxiety and Depression.	
	0-7 = Normal	
	8-10 = Borderline abnormal	
	11-21 = Abnormal	

Reference:

Zigmond and Snaith (1983)

Appendix IV – Presentation of data from this thesis

Oral presentations

- Skipworth RJE, Stewart GD, Bhana M, Christie J, Cronshaw AD, Fearon KCH, Ross JA. **A mass spectrometric approach to the discovery of protein biomarkers in the urine of patients with gastro-oesophageal cancer and cachexia.** Oral presentation at the *Society of Academic and Research Surgery Annual Conference*, Birmingham, January 2008.

- Skipworth RJE, Deans DAC, Brown DA, Hunter M, Breit SN, Ross JA, Fearon KCH. **Plasma levels of macrophage inhibitory cytokine-1 in patients with gastro-oesophageal cancer: association with systemic inflammation.** Oral presentation at the *Society of Academic and Research Surgery Annual Conference*, Birmingham, January 2008.

- Skipworth RJE, Acharyya S, Guttridge DC, Fearon KCH. **Deregulated dystrophin glycoprotein complex: a novel marker of cachexia and shortened survival in gastro-oesophageal cancer patients.** Oral presentation at the *Royal College of Surgeons of Edinburgh/College of Surgeons of Hong Kong Conjoint Scientific Congress*, October 2006.

- Skipworth RJE, Moses AGW, Sturgeon CM, Seth J, Ross JA, Fearon KCH. **Sex, systemic inflammation and survival in advanced pancreatic cancer.** Oral presentation at the *Society of Academic and Research Surgery Annual Conference*, Edinburgh, January 2006.

Poster presentations

- Ferriolli E, Skipworth RJE, Hendry PO, Scott A, Stenseth J, Dahele M, Wall L, Greig C, Fallon M, Preston T, Fearon KCH. **Physical activity: a responsive and meaningful patient-centred outcome measure in palliative oncology?** Poster presentation at 6th Research Congress of the European Association of Palliative Care, Glasgow, June 2010.

- Skipworth RJE, Stene GB, Hendry PO, Dahele M, Small AC, Blum D, Kassa S, Trottenberg P, Radbruch L, Strasser F, Preston T, Fearon KCH, Helbostad JL. **Patient-focused endpoints for clinical trials: criterion-based validation of accelerometer-based activity monitoring in advanced cancer.** Poster presentation at 6th Research Congress of the European Association of Palliative Care, Glasgow, June 2010.

- Skipworth RJE, Hendry PO, Dahele M, Small AC, Preston T, Fearon KCH. **Criterion-based validation of accelerometer-derived energy expenditure in advanced cancer.** Poster presentation at the *5th Cachexia Conference*, Barcelona, Spain, December 2009.

- Ferriolli E, Skipworth RJE, Stenseth J, Dahele M, Wall L, Greig CA, Fearon KCH. **Association between quality of life and directly monitored physical activity in patients with upper gastrointestinal cancer.** Poster presentation at the *5th Cachexia Conference*, Barcelona, Spain, December 2009.

- Skipworth RJE, Stewart GD, Bhana M, Christie J, Cronshaw AD, Fearon KCH, Ross JA. **A mass spectrometric approach to the discovery of protein biomarkers in the urine of patients with gastro-oesophageal cancer and cachexia.** Poster presentation at the *4th Cachexia Conference*, Tampa, USA, December 2007.

- Skipworth RJE, Deans DAC, Brown DA, Hunter M, Breit SN, Ross JA, Fearon KCH. **Plasma levels of macrophage inhibitory cytokine-1 in patients with gastro-oesophageal cancer: association with systemic inflammation.** Poster presentation at the *4th Cachexia Conference*, Tampa, USA, December 2007.

- Skipworth RJE, Moses AGW, Sangster K, Sturgeon CM, Seth J, Voss AC, Fallon MT, Anderson RA, Ross JA, Fearon KCH. **Interaction of gonadal status with systemic inflammation and opioid use in determining nutritional status and prognosis in advanced pancreatic cancer.** Poster presentation at the *4th Cachexia Conference*, Tampa, USA, December 2007.

- Acharyya S, Butchbach MER, Sahenk Z, Wang H, Saji M, Carathers M, Ringel MD, Skipworth RJE, Fearon KCH, Hollingsworth MA, Muscarella P, Burghes AHM, Rafael-Fortney JA, Guttridge DC. **Role of the dystrophin-glycoprotein complex in the pathogenesis of cancer cachexia.** Poster presentation at the *3rd Cachexia Conference*, Rome, Italy, December 2005. **Awarded Best Poster Prize**.

Appendix V – Publication of data from this thesis

Published abstracts

- Ferriolli E, Skipworth RJE, Hendry PO, Scott A, Stenseth J, Dahele M, Wall L, Greig C, Fallon M, Preston T, Fearon KCH. **Physical activity: a responsive and meaningful patient-centred outcome measure in palliative oncology?** *Palliative Medicine* 2010 June; **24**(4)(Supplement): S56-S57.

- Skipworth RJE, Stene GB, Hendry PO, Dahele M, Small AC, Blum D, Kassa S, Trottenberg P, Radbruch L, Strasser F, Preston T, Fearon KCH, Helbostad JL. **Patient-focused endpoints for clinical trials: criterion-based validation of accelerometer-based activity-monitoring in advanced cancer.** *Palliative Medicine* 2010 June; **24**(4)(Supplement): S63-S64.

- Skipworth RJE, Stewart GD, Bhana M, Christie J, Cronshaw AD, Fearon KCH, Ross JA. **A mass spectrometric approach to the discovery of protein biomarkers in the urine of patients with gastro-oesophageal cancer and cachexia.** *British Journal of Surgery* 2008 June; **95**(Supplement 4): 10.

- Skipworth RJE, Deans DAC, Brown DA, Hunter M, Breit SN, Ross JA, Fearon KCH. **Plasma levels of macrophage inhibitory cytokine-1 in patients with gastro-oesophageal cancer: association with systemic inflammation.** *British Journal of Surgery* 2008 June; **95**(Supplement 4): 12

- Skipworth RJE, Acharyya S, Guttridge DC, Fearon KCH. **Deregulated dystrophin glycoprotein complex: a novel marker of cachexia and shortened survival in gastro-oesophageal cancer patients.** *Surgical Practice* 2006 November; **10**(Supplement): A14.

- Skipworth RJE, Moses AGW, Sturgeon CM, Seth J, Ross JA, Fearon KCH. **Sex, systemic inflammation and survival in advanced pancreatic cancer.** *British Journal of Surgery* 2006 July; **93**(Supplement 3): 39-40.

Published manuscripts

- Skipworth RJE, Moses AGW, Sangster K, Sturgeon CM, Seth J, Voss AC, Fallon MT, Anderson RA, Ross JA, Fearon KCH. **Interaction of gonadal status with systemic inflammation and opioid use in determining nutritional status and prognosis in advanced pancreatic cancer.** *Supportive Care in Cancer* 2011 March; **19**(3): 391-401.

- Skipworth RJE, Stewart GD, Bhana M, Christie J, Sturgeon CM, Guttridge DC, Cronshaw AD, Fearon KCH, Ross JA. **Mass spectrometric detection of candidate protein biomarkers of cancer cachexia in human urine.** *International Journal of Oncology* 2010 April; **36**(4): 973-982.

- Skipworth RJE, Deans DAC, Tan BHL, Sangster K, Paterson-Brown S, Brown DA, Hunter M, Breit SN, Ross JA, Fearon KCH. **Plasma MIC-1 correlates with systemic inflammation but is not an independent determinant of nutritional status or survival in oesophago-gastric cancer patients.** *British Journal of Cancer* 2010 February; **102**(4): 665-672.

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- Stewart GD*, Skipworth RJE*, Pennington CJ, Lowrie AG, Deans DAC, Edwards DR, Habib FK, Riddick ACP, Fearon KCH, Ross JA. **Variation in dermcidin expression in a range of primary human tumours and in hypoxic/oxidatively stressed human cell lines.** *British Journal of Cancer* 2008 July; **99**(1): 126-132. * = joint first authors

- Stewart GD, Skipworth RJE, Ross JA, Fearon KCH, Baracos VE. **The dermcidin gene in cancer: role in cachexia, carcinogenesis and tumour cell survival.** *Current Opinion in Clinical Nutrition and Metabolic Care* 2008 May; **11**(3): 208-213.

- Eley HL, Skipworth RJE, Deans DAC, Fearon KCH, Tisdale MJ. **Increased expression of phosphorylated forms of RNA-dependent protein kinase (PKR) and eukaryotic initiation factor 2α (eIF2α) may signal skeletal muscle atrophy in weight-losing cancer patients.** *British Journal of Cancer* 2008 January; **98**(2): 443-449.

- Skipworth RJE, Stewart GD, Dejong CHC, Preston T, Fearon KCH. **Pathophysiology of cancer cachexia: much more than host-tumour interaction?** *Clinical Nutrition* 2007 December; **26**(6): 667-676.

- Wieland B, Stewart GD, Skipworth RJE, Sangster K, Fearon KCH, Ross JA, Reiman TJ, Easaw J, Mourtzakis M, Kumar V, Pak BJ, Calder K, Filippatos G, Kremastinos DT, Palcic M, Baracos VE. **Is there a human homolog to the murine proteolysis–inducing factor?** *Clinical Cancer Research* 2007 September; **13**(17): 4984-4992.

- Skipworth RJE, Stewart GD, Ross JA, Guttridge DC, Fearon KCH. **Molecular mechanisms of skeletal muscle wasting: implications for therapy.** *Surgeon* 2006 October; **4**(5): 273-283.

- Stewart GD, Skipworth RJE, Fearon KCH. **Cancer cachexia and fatigue.** *Clinical Medicine* 2006 March/April; **6**(2): 140-143.

- Acharyya S, Butchbach MER, Sahenk Z, Wang H, Saji M, Carathers M, Ringel MD, Skipworth RJE, Fearon KCH, Hollingsworth MA, Muscarella P, Burghes AHM, Rafael-Fortney JA, Guttridge DC. **Dystrophin glycoprotein complex dysfunction: a regulatory link between muscular dystrophy and cancer cachexia.** *Cancer Cell* 2005 November; **8**(5): 421-432.